

A NEW TYPE OF HEREDITARY HARELIP IN THE HOUSE MOUSE

S. GLUECKSOHN-SCHOENHEIMER AND L. C. DUNN

Department of Zoology, Columbia University

SIX FIGURES

Harelip and cleft palate have been reported several times in mice as recessive mutations with variable and usually asymmetrical expression (see Grüneberg, '43, for review) and their occurrence in man is well known and in many cases has been shown to be due to a gene with variable expression (Grünberg, '13, Steiniger, '39). A new occurrence of this condition in a strain of mice in our laboratory differed in several respects from those previously reported: the harelip was very marked, median, symmetrical and regular; it acted as a lethal, all young dying shortly after birth; it appeared in one family only, as though due to a peculiar combination of a gene with a particular set of other genetic factors.

The new harelip character was first found in 18 out of 42 offspring of a brother-sister mating in a stock which had been sent to us by Mr. F. G. Carnochan of Carworth Farms, New City, New York. Mr. Carnochan had noted in his standard inbred albino line C. F. a new morphological character "pug nose" and in October 1943 sent us several specimens of "pug" for genetical study. "Pug" was distinguished by a short face, due probably to shortened nasal bones and premaxillae but it was quite variable in expression and showed intergradation with normal. The few observations we were able to make before the stock was lost showed that matings of pug by pug produced both pug and normal offspring (28 pug, 13 normal) as did matings of pug male with normal sis-

ters (9 pug, 10 normal) whereas outcrosses of pug male by unrelated normal females yielded only normal offspring (18). This was consistent with the assumption that "pug" animals contained a recessive mutation which did not always express itself, although difficulties in classification prevented any conclusive analysis. In the second generation descended from a pair of pug animals received from Mr. Carnochan we mated two pug males with their two sisters which did not show the pug character. This mating produced 4 litters containing 20 normal, 4 pug and 18 offspring with very marked median harelip and cleft palate. None of the latter was able to nurse; all died within a day after birth and were preserved for study. The normal sibs of the harelip animals were inbred for two further generations before the stock was lost, but no further occurrences of harelip were found among the offspring. Although it is clear from the above that a new genetic factor was present in the harelip animals, we cannot ascribe its inheritance nor its relation to the "pug" character. The ratio of 20 normal to 22 abnormal (4 pug, 18 harelip) suggested that harelip might be due to a mutant gene, which produces harelip when combined with pug. The absence of harelip offspring in other matings of "pug" by "pug" makes it unlikely that "harelip" was simply the homozygous expression of a mutation which when heterozygous produced, with occasional overlaps to normal, the pug character.

Because of the unusual features of this new type of harelip, 17 of the 18 specimens are described below from skulls fixed, stained with Alizarin Red S and cleared according to the method of Lipman ('35) slightly modified for our purposes.

In plate 1 the skull of a normal newborn (1) is compared with two harelip litter mates from litter 635. Of the latter the abnormal mouse at the left (fig. 2) showed at birth harelip and cleft palate; clearing and staining of the skull revealed the following additional abnormalities: nasals and frontals were wide apart and in addition nasals and premaxillae were considerably shortened. The harelip skull is

thus shorter and wider than that of its normal litter mate. The ventral aspect of the abnormal skull was studied after removal of the mandibles; the main abnormalities were observed in the region of the palatine. The palatine bones and the palatine processes are small and not fused — thus causing the cleft palate. The second abnormal skull in this litter (fig. 3) shows similar abnormalities but to a lesser degree; dorsally, nasals and frontals are slightly shortened and not completely fused, while the ventral picture resembles closely that of the first skull with palatine bones and processes small and not fused. The third skull in this litter — not illustrated — shows an even more extreme condition than the first one: nasals, frontals and parietals are wide apart and nasals and premaxillae are shortened so that the skull again is shorter and wider than normal. Ventrally, we find — as in the two skulls described previously — a cleft palate which gives the appearance of a bilateral cleft due to the vomer which extends into the broad defect in the roof of the mouth.

In another skull from a different litter with harelip abnormality (not illustrated) the nasals, frontals and parietals are far apart while ventrally there is a broad cleft in the roof of the mouth which appears cut into two by the vomer.

The fifth skull in this series from a litter mate of the previous animal is quite an extreme case, with nasals, frontals and parietals widely apart and nasals and premaxillae shortened. Ventrally, there is a complete cleft extending from the roof of the mouth through the nasals.

The next group of 6 newborn mice with harelip abnormalities all belonged to the same litter. The first 4 skulls are fairly uniform in the extent of their malformations. They have only a slight harelip and the degree of non-fusion of nasals, frontals and parietals is small. In all 4 specimens nasals and premaxillae are shortened and the skulls have a pug-nose appearance. Ventrally, all 4 skulls show the typical cleft palate with the vomer extending into the free space at the roof of the mouth. In the 5th specimen in this litter the harelip is only slight and the palate is not cleft. Dorsally,

the only abnormality found is a slight amount of non-fusion of the frontals.

The 6th skull is again quite abnormal with an extreme harelip and a considerable gap between nasals and frontals; the parietals also are not fused completely. The ventral aspect of this skull shows in addition to the extensive harelip a cleft palate with the vomer extending into the cleft.

The next and last group of abnormals consists of 6 harelip skulls all of which appeared in *one* litter (litter 654). The 6 skulls are fairly uniform in the extent of their abnormalities and all six are extremely malformed. Figures 4, 5 and 6 show examples of abnormal skulls in this group: in addition to the actual harelip, the nasals, frontals and parietals show a very wide gap between them. Nasals and premaxillae are shortened, giving the head the typical "pug-nose" appearance. From a ventral view the skulls again show in addition to the extreme harelip an extensive cleft palate into which the vomer extends as though cutting it in half (fig. 6).

In summarizing the malformations found in the skulls of mice described as "harelip" at birth we find the following features common to all of them:

1. At birth these abnormals are recognized by the presence of a cleft in their upper lip — which classifies the abnormality as harelip. Also, a cleft in the palate is found in all of them but one. Furthermore, the heads are shorter and wider than normal and give the animal a "pug-nose" appearance.

2. The cleared and Alizarin Red S-stained specimens show varying degrees of non-fusion of the following cranial bones: nasals, frontals, parietals. The extent of non-fusion of these bones varies from one specimen to another as appears in the photographs in this paper. The "pug-nose" appearance of the newborns is borne out by the observations on these cleared skulls: nasals and premaxillary bones are shortened considerably and the width of the skull is increased relatively if not absolutely.

3. The ventral aspect of the skull reveals a cleft palate characterized by failure to close of the constituents of the hard palate, i.e., the palatal bones, the palatal processes of the maxillary bones and the premaxillary bones. The vomer is seen to extend typically into this cleft cutting it in half.

The new condition described thus represents a syndrome of abnormalities of the head consisting of harelip, cleft palate, parted nasals, frontals and parietals and shortening and widening of the skull. This latter feature results in the "pug-nose" appearance of the abnormal young.

In attempting to classify the malformations described here according to the terminology used by Grünberg in his chapter on malformations of the head in Schwalbe's *Morphologie der Missbildungen des Menschen und der Tiere*, the closest approximation of a human malformation to those described here seems to be the bilateral complete cleft of lip, alveolus and palate. The new syndrome differs in morphology from the harelip mice described by Reed and Snell ('32). In their case, a bilateral cleft extends through the upper lip, upper jaw and hard palate *lateral* to the incisors — while in our cases the cleft is always median, passing between the incisors. Steiniger's cases seem to resemble Reed and Snell's more than those described here.

The genetical behavior and expression of the several harelip conditions described in mice are quite irregular. The most extensive observations are those of Steiniger ('39) who showed that in matings which produce harelip, the proportion of harelip young decreases with increasing age of mother and increasing parity of litter, thus proving that the genetic constitution is not solely responsible for the appearance or suppression of the character. It is perhaps because of this fact that no conclusive genetic analysis of harelip in mice has been made.

In our case, 18 harelip young were produced in a short space of time from matings of one set of siblings in one stock. The shortening of the skull of the harelip young indicates that this harelip syndrome develops upon and per-

haps takes its peculiar expression from the "pug" or short skull character which has been found only in this stock. Its high frequency in these litters rather speaks against ascribing it to a newly arisen recessive, but suggests that a combination of a special genotype and a peculiar set of conditions were responsible for its appearance.

LITERATURE CITED

- GRÜNBERG, KARL 1913 Die Missbildungen des Kopfes, in E. Schwalbe: Die Morphol. d. Missbild. d. Menschen u. der Tiere. 3. Teil, 4. Kap. pp. 113-204.
- GRÜNEBERG, HANS 1943 The Genetics of the Mouse. University Press, Cambridge.
- LIPMAN, HARRY J. 1935 Staining the skeleton of cleared embryos with Alizarin Red S. *Stain Technology* 10: 61.
- REED, S. C. AND G. D. SNELL 1931 Harelip, a new mutation in the house mouse. *Anat. Rec.* 51: 43-50.
- STEINIGER, F. 1939 Neue Beobachtungen an der erblichen Hasenscharte der Maus. *Zeitschr. f. menschl. Vererb. u. Konstitutionslehre*, 23: 427-462.

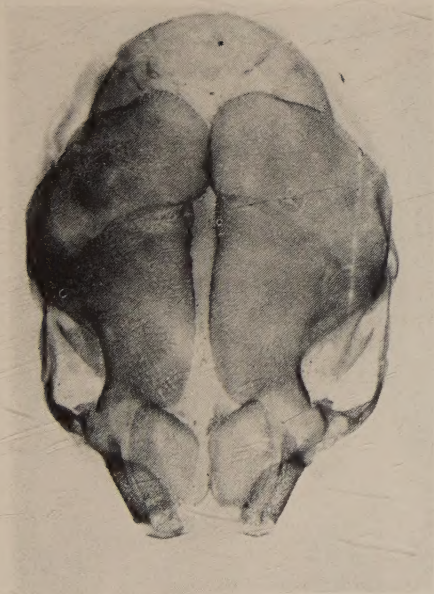
PLATE 1

EXPLANATION OF FIGURES

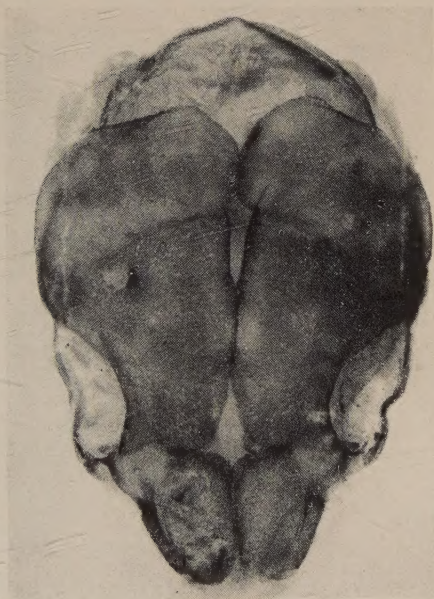
- 1 Dorsal view of cleared skull of newborn normal mouse stained with Alizarin Red S. 8 ×.
- 2 Dorsal view of cleared skull (635A) of harelip mouse stained with Alizarin Red S. Frontals and nasals are parted; note shortening of nasals and premaxillae causing the "pug-nose" appearance of the skull. 10 ×.
- 3 Dorsal view of cleared skull (635B) of harelip mouse stained with Alizarin Red S. Frontals slightly parted. 10 ×.



1



2

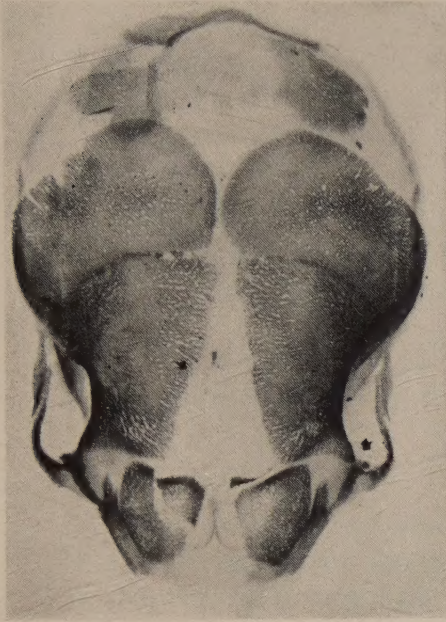


3

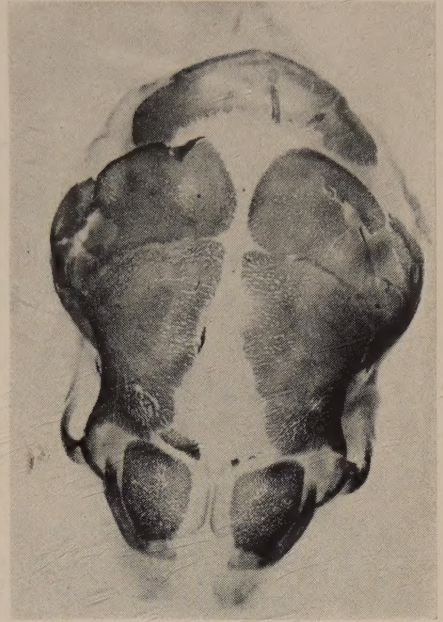
PLATE 2

EXPLANATION OF FIGURES

- 4 Dorsal view of cleared skull (654C) of harelip mouse stained with Alizarin Red S. Parietals, frontals, nasals widely parted. 9 ×.
- 5 Dorsal view of cleared skull (654D) of harelip mouse stained with Alizarin Red S. Parietals, frontals, nasals widely parted. 8 ×.
- 6 Ventral view of cleared skull (654D) of same harelip mouse as in figure 5. Vomer can be seen through cleft in hard palate. 7 ×.



4



5



6

THE FUNCTIONAL SIGNIFICANCE OF THE CARDIAC JELLY IN THE TUBULAR HEART OF THE CHICK EMBRYO

ALEXANDER BARRY

Department of Anatomy, University of Michigan Medical School

TWO FIGURES 1*

When, at about the 40th hour of incubation, the heart of the embryonic chick begins to pump blood it is essentially a simple tubular structure, looped to the right. Its wall consists of a tube of endothelium within a sleeve of epimyocardium. In living as well as in fixed preparations there is a space between these two layers. In the living condition this space appears optically homogeneous. In fixed preparations it is found to be filled with a delicate non-cellular meshwork of fibrils so fine as to be scarcely visible under the oil immersion lens. Davis ('24) named this material cardiac jelly and demonstrated its gelatinous character *in vivo*. Its composition and structure in the living condition is still problematical. The present report is not primarily concerned with the composition of the cardiac jelly, but rather with its role in the functioning of the primitive tubular heart.

In the heart of a chick embryo of 40 hours incubation, the cardiac jelly surrounds the endothelium of that part of the heart which is destined to form the ventricle and truncus of later stages (Patten, '22). In cross section this sheath of cardiac jelly is not of equal thickness. It is distinctly thinner at the primary ventral and dorsal midlines. Cephalically it extends to the aortic roots, and caudally to the atrial region of the heart. In the fixed heart of a chick of this age, there

is no marked regional thickening along the length of the cardiac tube. Thus, if the heart is to pump blood, the mechanism could not be other than the peristaltoid type of contraction, the nature of which is so clearly shown by the superimposed tracings from the Patten, Kramer films ('33). This means that the blood is propelled by a wave of contraction sweeping from the sino-atrial end of the heart to its truncus end. The presence of the relatively thick sheath of cardiac jelly might at first appear needlessly cumbersome in a heart of this type. However, an analysis of the mechanics of a peristaltoid beat makes it clear that the cardiac jelly is a requisite for effective pumping action.

To analyze the role of the cardiac jelly, let us assume a simplified tubular heart of circular cross section, and attempt to infer what its action would be if a layer of cardiac jelly were not present, that is to say, if the contractile sleeve of myocardium were in direct contact with the endothelium. If such a heart is to propel blood in a peristaltoid manner, then its lumen must be practically obliterated at systole. Strictly speaking a complete closure of the lumen would probably not be necessary, since the blood pumped is not a homogeneous fluid, but a suspension of cells. Thus, if the diameter of the lumen at systole were not appreciably larger than the diameter of a red blood cell ($20\ \mu$) it is likely that the corpuscles would pile up in the systolic constriction, and effectively prevent regurgitation.

Since in attempting the analysis of its action we are assuming that the heart is circular in cross section, we may let R represent the radius of the myocardium, and C its circumference. The subscripts s and d may be used to designate these dimensions in systole and diastole respectively. The myocardium of the embryonic heart behaves like most contractile tissues in that its systolic shortening does not exceed a certain proportion of its diastolic length. In living hearts of this stage of development studied in situ, this degree of shortening is approximately 20%. The myocardium can be seen to shorten longitudinally as well as circumferentially, and will

be assumed to do so to the same degree in the following analysis:

Thus, $C_s = .8 C_d$, (1)

hence $2 \pi R_s = .8 (2 \pi R_d)$

and, $R_s = .8 R_d$ (2)

	Large Diameter	Small Diameter
Diastole		
Systole		
Systolic closure	None	Good
Stroke volume	Large	Minimal

Fig. 1 Diagramatic cross sections of hypothetical tubular hearts without cardiac jelly in their walls. The two pairs of diagrams represent hearts of large and small diameters-respectively. The systolic sections are drawn on the basis of a 20% shortening of the circumference on contraction. Note that when the diastolic diameter is large, the change in cross sectional area, (and hence in the stroke volume) is also large, but that the lumen does not close in systole. When the diastolic diameter is sufficiently small to allow systolic closure, the concomitant stroke is negligible.

From this it is clear that if the systolic radius of the lumen were to be only 20μ , the diastolic radius would be 25μ , and hence the heart would scarcely dilate on diastole, and could pump no significant amount of blood. Carrying this line of thinking a step farther, it is evident that in a tubular heart, the stroke volume will be determined, in part, by the diastolic radius of the lumen. Thus, if an appreciable quantity of blood

is to be pumped, the diastolic radius must be relatively large, but if this is the case, the systolic lumen will be too large to permit adequate closure for pumping action.

It appears, therefore, that if a tubular heart is to operate effectively, there are two requirements to be met. First, the radius of the epimyocardial sleeve must be relatively large if the heart is to have a significant stroke volume. Second, the lumen of the heart on systole must be nearly zero. A simple means of meeting this problem would be to allow the myocardium to be of relatively large radius, but to interpose between it and the endocardium a layer of material of such character that it would transmit the force of contraction radially down against the endothelium. This material would have to be firm enough to squeeze shut the lumen, and yet resilient enough so that it would not require an undue proportion of the energy of contraction in being so deformed. Obviously this layer could not be fluid in character, since if it were, the force of contraction would be transmitted along the entire length of the endothelial tube, and would cause no localized constriction. In the tubular heart of the young chick embryo we have exactly the type of construction suggested above, with a layer of resilient cardiac jelly lying between the epimyocardium and the endothelium.

Let us now modify the simplified tubular heart postulated above, by interposing a layer of cardiac jelly in its wall. By simple geometrical analysis we can make a rough estimate of how thick such a layer should be, if the heart were to function properly.

If we let R be the radius of the myocardial sleeve, r the radius of the lumen, and t be the thickness of the cardiac jelly at diastole, then:

$$R_d = r_d + t \quad (3)$$

Let us take a segment of this tubular heart of unit length at diastole. The volume of the cardiac jelly will be:

$$V_d = \pi R_d^2 - \pi r_d^2 \quad (4)$$

The volume of the cardiac jelly of this segment at systole, assuming that both the length and circumference of the myocardium have shortened to 80% of their diastolic value, will be:

$$V_s = .8 \pi R_s^2 - .8 \pi r_s^2 \quad (5)$$

As has been shown previously, the systolic radius (r_s) requisite for an effective peristaltoid beat must be extremely small. For the sake of simplicity in formulation, this value (r_s) will be taken as zero, since the error introduced by this assumption is negligible in the following analysis.

Therefore, if $r_s = 0$

Then equation (5) becomes $V_s = .8 \pi R_s^2$

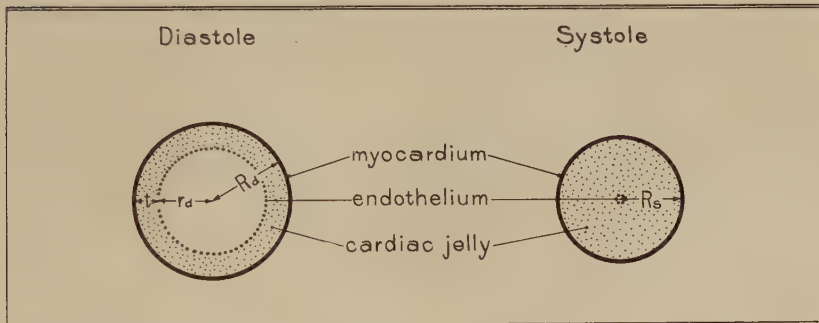


Fig. 2 Diagrammatic cross sections of a tubular heart with cardiac jelly in its wall, showing its dimensions in the diastolic and the systolic condition. The diagrams are drawn on the basis of a 20% shortening of the circumference on systole. The dotted line indicates the endothelium, the solid line, the myocardium, and the cardiac jelly is indicated by stippling.

t = thickness of the cardiac jelly layer in diastole

r_d = radius of the lumen in diastole

R_d = radius of the myocardial sleeve in diastole

R_s = radius of the myocardial sleeve in systole

If the cardiac jelly is essentially incompressible, then its systolic volume will be equal to its diastolic volume:

$$\pi (R_d^2 - r_d^2) = .8 \pi R_s^2$$

thus

$$R_d^2 - r_d^2 = .8 R_s^2 \quad (6)$$

However

$$R_s = .8 R_d \quad (\text{from equation 2})$$

hence

$$R_s^2 = .64 R_d^2$$

and	$R_d^2 - r_d^2 = .8 (.64 R_d^2)$	(from equation 6)
hence	$R_d^2 - r_d^2 = .512 R_d^2$	
hence	$r_d^2 = .488 R_d^2$	
Since	$r_d = \sqrt{.488} R_d = .69 R_d$	
	$R_d = r_d + t$	(equation 3)
then	$r_d = .69 (r_d + t)$	
hence	$r_d - .69 r_d = .69 t$	
hence	$0 - 31 r_d = 0.69 t$	
hence	$r_d = 2.2 t$	
or	$t = 0.45 r_d$	

It is clear that in such a heart as we have been considering the deformation of the cardiac jelly will require a certain proportion of the energy of the myocardial contraction, and this portion cannot, therefore, be utilized in the propulsion of the blood. Such an apparent loss of energy seems to make the tubular heart an inefficient pump. However, the situation is not as wasteful as it appears. If we consider the status of the cardiac tube at the height of systole, we see the myocardium contracted, the layer of cardiac jelly deformed and squeezed down upon the endothelium so that the lumen is obliterated. When the myocardial sleeve relaxes during diastole, the layer of cardiac jelly, due to its own resiliency, springs back to its original shape, pulling the endothelium with it, and reestablishing a lumen in the heart. This reopening of the cardiac lumen will tend to suck blood into the heart. The blood will necessarily come from its venous end, since at the time of diastole of the atrial end of the tube, the truncus end is still in systole, effectively preventing regurgitation of blood from the aortic roots. Thus the energy of myocardial systole is in part used to force blood out of the heart into the arterial system, the rest being used to compress the resilient cardiac jelly, (i.e., being transformed into potential energy). This portion of the energy of each systole reappears during the subsequent diastole as mechanical energy in refilling the

heart for the following systole. The tubular heart may be considered to be very similar in action to an aspirator bulb. When the bulb is squeezed, part of the energy is used to expel the contents under pressure, and part to deform the rubber of the bulb. When the force is relaxed, the bulb springs back to its original shape, its stored potential energy being released and used in refilling its lumen.

This hypothesis can be substantiated by direct observations. The tubular heart of a chick of about 40–48 hours incubation age observed *in vivo*, is sufficiently transparent to allow one to see the blood within its lumen. The contents of the lumen disappear on systole as the blood is forced out and the endothelial walls come into contact. If one transsects the body of such an embryo caudal to the sinus venosus, the venous return pressure to the heart will drop to the ambient pressure. Thus there is no pressure to force blood into the heart, and yet extravasated blood can be seen to be sucked into the heart during subsequent diastoles. This observation is understandable only on the basis of the sucking action produced by the heavy component of cardiac jelly in the wall of the heart at this stage of development.

By the 44th hour of incubation, the ventricular portion of the heart has been thrown into a loop, but the essential structure of its wall is unchanged. The truncus remains as a narrow tube, with a relatively heavy layer of cardiac jelly in its wall. Its lumen is rather narrow even in diastole. At this stage of development the fixed truncus does not have any localized thickenings of cardiac jelly along its length. Thus its entire wall must contract on systole sufficiently to close the lumen if it is to act in preventing regurgitation of blood into the ventricle. It must act in a manner comparable to that described above for the entire “primitive” tubular heart.

When the ventricle contracts, it ejects a gout of blood through the narrow truncus, dilating its lumen. One can deduce that some energy will be lost in this process. However, the truncus wall presents its minimal resistance to stretching at this phase, since the cardiac jelly of its wall is ensheathed

by myocardium in *diastole*. As the blood passes through the truncus into the aortic arches, the pressure on the aortic side will increase. When the ventricle relaxes, its pressure drops to a low value. At this time the difference between the aortic and the ventricular pressures (reguritation pressure) will be maximal. This, then, will be the time when the valve action of the truncus is subjected to its greatest strain. However, it is precisely at this time, immediately after ventricular systole, that the contraction wave has swept to the truncus. Therefore its wall presents its greatest closing force, since its wall consists of cardiac jelly reinforced by myocardium in *systole*.

This type of valve action, although effective in preventing the regurgitation of blood, is relatively inefficient since its operation depends upon myocardial contraction, an energy consuming process. It also is inefficient in another respect. Since the ventricle must eject its blood through a narrow opening there must be considerable energy loss due to friction. Yet if the lumen of the truncus were larger, the contraction of its myocardial coat could not squeeze it shut against regurgitation.

In later developmental stages, when the endocardium of the truncus heaps up into endocardial cushion tissue pads, and as these pads gradually are molded to the definitive semilunar valves, a new mechanism of closure of the arterial outlet of the heart is introduced. By the time true valve flaps are present, the ventricles empty themselves through a relatively large orifice, and the thin valves fold back against the wall of the outlet. When blood starts to regurgitate at the end of ventricular systole, the flaps are caught in the backflow, and swing across the lumen of the outlet, blocking it completely. This mechanism has two marked advantages over the primitive type of closure discussed above. First, the ventricle can empty itself through a reasonably large opening, thus reducing frictional losses. Second, regurgitation is prevented by the strength of the connective tissue of the valve flaps

rather than by the contraction of myocardial tissue, and therefore, a considerable amount of muscular energy is saved.

The change from the first to the second type of valvular mechanism takes place gradually. During the intermediate stages one sees a progressive decrease in the myocardial functional component, and a corresponding increase in the connective tissue component of the truncus closure.

It is of interest to consider some of the developmental implications of the fact that in a tubular heart the thickness of the layer of cardiac jelly must be about 45% of the radius of the lumen in diastole if the heart is to propel blood with a peristaltoid beat. It is well known that the mass of the growing embryo increases rapidly, and it is axiomatic that the minute volume output of the heart must increase at a corresponding rate if the tissues are to receive an adequate blood supply. The minute volume of the heart is a product of the rate and the stroke volume. After the 50th hour the rate of the heart increases relatively slowly (Barry, '40). Therefore, much of this necessary increase in minute volume output must be accounted for by an increase in stroke volume. This requires a marked increase in the diastolic radius of the heart's lumen, which would necessitate a proportionate increase in the thickness of the cardiac jelly. It seems that if this were the case, an increasingly larger proportion of the energy of myocardial systole would be required to deform the cardiac jelly, and therefore less would be available for the propulsion of the blood. This situation is complicated by the fact that as the vascular bed of the embryo increases in extent, the aortic pressure must increase to maintain an adequate circulation through its increased resistance. Also as the diameter of the heart increases, the circumferential stress on its wall will increase in direct proportion even if the pressure remains constant. As a result of these two supplementary facts the entire wall must increase in strength (and therefore in thickness), to withstand the stress imposed upon it by its own greater diameter and by the greater systolic pressure.

All of these factors lead one to predict that if the tubular heart with a peristaltoid beat were to persist as the heart of the developing embryo it would have to become progressively more massive and less efficient until it reached a stage at which it could no longer meet the requirements of the embryo. In the present state of our knowledge we cannot profitably attempt to compare the "efficiency" of embryonic with adult myocardium as contractile tissues. Nevertheless, it is of interest that the changes in the configuration and the arrangement of tissues in the embryonic heart are such that we see a marked increase in its efficiency as a pumping mechanism, even if we assume no increase in the physiological efficiency of the contractile component itself.

LITERATURE CITED

- BARRY, A. 1940 Age changes in the pulsation frequency of the embryonic chick heart. *Jour. Exp. Zool.*, 85: 157-170.
- DAVIS, C. L. 1924 The cardiac jelly of the chick embryo. *Anat. Rec.*, 27: 201-202.
- PATTEN, B. M., 1922 The formation of the cardiac loop in the chick. *Am. J. Anat.*, 30: 373-397.
- PATTEN, B. M., AND T. C. KRAMER 1933 The initiation of contraction in the embryonic chick heart. *Am. J. Anat.*, 53: 349-375.

VALVULAR ACTION IN THE EMBRYONIC CHICK HEART BY LOCALIZED APPPOSITION OF ENDOCARDIAL MASSES

BRADLEY M. PATTEN, THEODORE C. KRAMER AND
ALEXANDER BARRY

Department of Anatomy, University of Michigan Medical School

SIX FIGURES

The heart of a chick embryo at the time the blood first starts to circulate through it is essentially a double-layered tube. Its endocardial layer consists of an endothelial lining held in place within the outer epimyocardial investment by a surprisingly thick layer of gelatinous, non-cellular material which Davis ('24, '27) called cardiac jelly. Blood enters this primitive young heart at its caudal end and is forced out its cephalic end by peristaltoid contractions of the myocardium. The fact that the cardiac tube soon becomes bent on itself does not at first essentially change the way the blood passes through its unchambered length. Nor does this early tubular heart have anything resembling valves as we know them in the adult heart. It is not surprising, therefore, that when the recently established circulation is studied in living embryos one sees a very jerky progression of the corpuscles in the vessels entering and leaving the heart. There is with each cardiac cycle what one might call a "backlash." This term in this connection is meant to convey the idea that toward the end of each contraction cycle the corpuscles in an artery lose a little of the progression they have made under the thrust of systole. In veins a similar backlash occurs as contraction commences at the atrial end of the cardiac tube.

Early in the third day of incubation the corpuscles begin to progress with less and less jerkiness. The change is so gradual that one hardly notices it in watching the same embryo through the transition period. If, however, an embryo after the change, is directly compared with one before the change has occurred, the contrast is quite striking. In the older embryo, between each beat, the corpuscles seem to coast to a momentary pause without any of the retrogression seen in younger embryos. Their whole progression appears smoother, and the nature of their movement suggests the operation somewhere in the system of a valvular mechanism guarding against back flow. Yet, as already emphasized, there is no structure in the embryonic heart at this age which even remotely resembles the usual flap-like type of valve seen in the adult or in older embryos. Careful restudy of films recording the cardiac activity at the critical stages of development first suggested the answer to the change in character of blood movement. At the level of transition from atrium to ventricle, "slow-motion" pictures showed the endocardium in the atrioventricular canal heaping up in "mounds" so that at the close of each contraction cycle the mounds made contact with each other and completely blocked the cardiac lumen. During this period of closure careful study of both film records and living embryos revealed no back flow of blood from the contracting ventricle into the atrium.

The non-committal term endocardial mounds is used in describing this phenomenon rather than endocardial cushions because at the ages when the closure first becomes apparent the material which is heaped up is non-cellular cardiac jelly (plate 1, A). During the third day cells grow into the cardiac jelly and it gradually becomes converted into a plastic young connective tissue of the type commonly described as endocardial cushion tissue. Contrary to the conclusions of Chang ('32, p. 225), the source of the cells involved appeared to us to be the endothelium. Careful scrutiny of the endothelial lining in embryos just before cellular invasion of the cardiac jelly is due to commence reveals occasional local doubling of the

ordinarily single cell layer (see place indicated by arrow in plate 1, A). The picture suggests a recent cell division with one of the cells moving out of the endothelial layer toward the cardiac jelly. Slightly older embryos present conditions consonant with such an interpretation. Sprawling mesenchymal cells can be seen in the cardiac jelly adjacent to the endothelium, whereas none have as yet reached the cardiac jelly adjacent to the myocardium (plate 1, B). A well-graded series of embryos is necessary if this intermediate stage is to be observed for once the cellular invasion has started it spreads rapidly all the way through the cardiac jelly to the myocardium (plate 1, C). One gets the impression from seeing a series of fixed preparations that the cardiac jelly serves as a supporting medium through which these cells move. It should be emphasized, however, that the lace work of strands along which the cells appear to be moving undoubtedly is a distorted representation incident to the coagulation of a substance which in the living embryo was gelatinous and more nearly homogeneous. Further studies are being undertaken in an effort to learn more of its true physical and chemical properties.

While this alteration in endocardial histology is occurring, study of living embryos shows relatively little change in the manner in which closure of the cardiac lumen is effected by the mounds. As endocardial cushion tissue replaces the cardiac jelly, the mounds become more sharply circumscribed and the closure is perhaps a little more positive, but the essential mechanism of blocking the cardiac lumen against regurgitation by the heaping up of a mound of plastic material remains unchanged.

This curious action of the endocardial mounds was noted in some of our earliest films which were made between 1928 and 1930. At about that time it was pointed out to Dr. Bremer on the occasion of a visit he made to us in Cleveland, where this work was begun. Dr. Bremer subsequently, in his own material, confirmed these early observations (personal communication) and mentioned them in connection with his work

on circulatory disturbances in chick embryos (Bremer, '32b, p. 277). We know of nothing on the subject in the literature other than Bremer's brief comment and a surmise by Fischel ('29, p. 698) with reference to much older embryos to the effect that the endocardial cushions of the atrioventricular canal might be apposed to each other during systole and so exert a valvular action. Repeatedly, since it was first noticed, we have seen this same phenomenon both in living embryos and in micro-moving picture records of embryonic heart action. It has been mentioned in lectures given in connection with the showing of our films, but as far as we are aware, it is not a generally recognized phenomenon and has never been described in any detail. The writing up of these observations has been delayed pending the securing of additional film which would show more clearly certain phases of the changes involved. Some of our recently obtained records seemed to meet this need and led to the publication of this long postponed account.

Aside from seeing the living heart, or moving pictures of it in action, probably the easiest way of understanding the manner in which these endocardial mounds operate is afforded by superimposing tracings made by projecting through a photographic enlarging apparatus frames of a moving picture film showing different phases of the action. Such tracings showing the endocardial mounds in their open and closed position at the level of the atrioventricular canal in the heart of a chick of about 50 hours of incubation are reproduced in figure 1. The embryo was mounted so it could be viewed from the left, an orientation which at this age gives the best exposure of the atrioventricular canal. The solid outline represents the external contour of the heart in diastole. The broken lines show the external configuration during systole. The positions of the endocardial mounds, separated as they appear during the filling of the ventricle, are indicated by the beaded lines. The positions of the endocardial mounds as they are apposed, blocking the atrioven-

tricular canal against regurgitation of blood, are indicated by dotted lines.

This peculiar type of valvular action was noticed first at the atrioventricular canal. But as soon as the heart action was restudied in the light of this finding it became apparent that there was a second region where closure was effected by a similar mechanism. This was at the transition from ventricular conus to truncus arteriosus — in other words, at

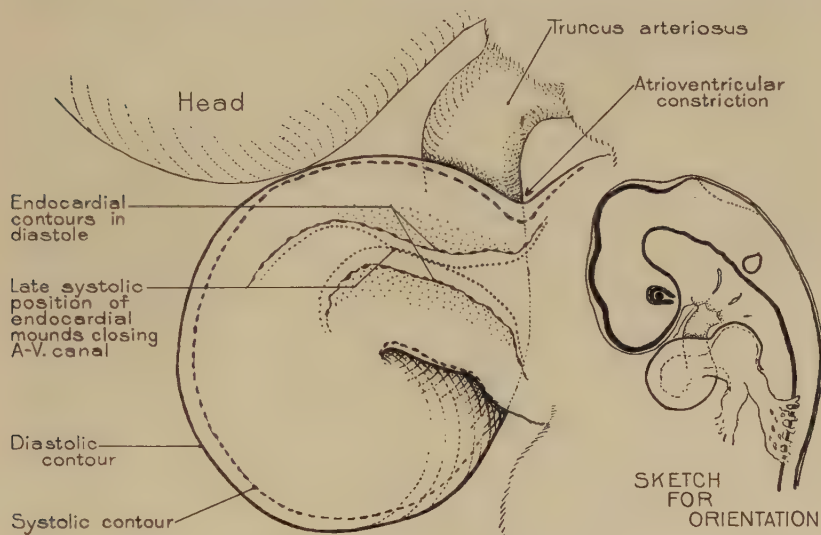


Fig. 1 Superimposed tracings made by projecting frames from moving picture film showing the heart action of a 50-hour chick. The solid external outlines and the beaded endothelial contours show conditions when blood is passing through the atrioventricular canal. The broken external outline and the dotted endothelial contours show conditions when the endocardial mounds are apposed to each other, blocking the lumen against back flow.

approximately the level where the semilunar valves of the aorta and the pulmonary trunk are destined to be formed. It is interesting that this curious primitive type of valvular action should be manifested at this particular level even before the truncus is partitioned into aortic and pulmonary outlets and, of course, long before the button-like endocardial cushion tissue primordia of the leaflets of the semilunar valves are

as much as suggested morphologically. This situation at the ventricular outlet is, however, in line with what we have seen happening at the atrioventricular canal. There an effective closure occurs before the endocardial masses that later divide the canal into right and left channels have acquired the ingrowing cells which give them their characteristic histological structure. In this location, also, anything presaging the formation of the definitive flap-like atrioventricular valves lies far in the future, speaking developmentally.

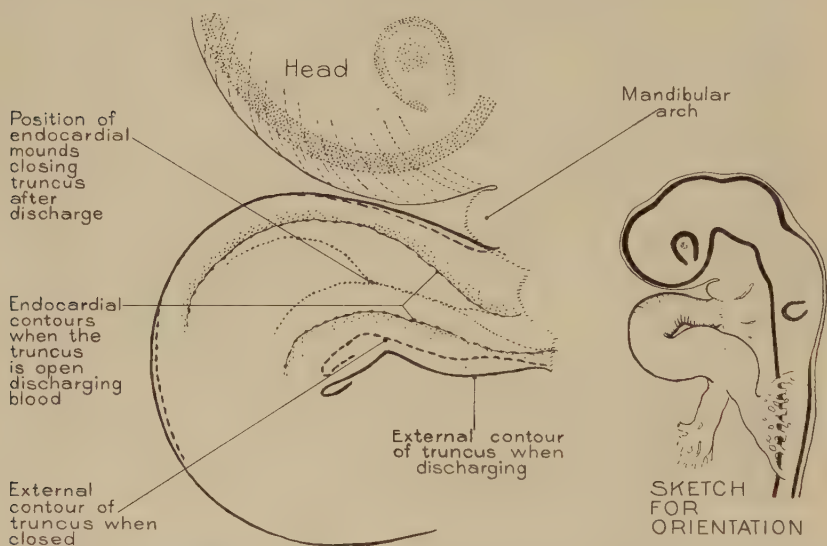


Fig. 2 Superimposed projection tracings showing closure by endothelial mounds at the transition from conus to truncus arteriosus. Scheme of representation similar to that employed in figure 1.

The closure at the base of the truncus arteriosus can readily be seen in a chick of about 48 to 50 hours. (Age scale as indicated in Patten, B. M., '22.) At this age there is considerable individual variability as to how tight the cardiac loop is twisted. An embryo in which the loop is rather loose, as indicated in the orienting diagram in figure 2, leaves the truncus projecting free of the body and in favorable position for observation and photography. The outlines of the truncus shown in figure 2 were made in the same manner as those of

the atrioventricular canal shown in figure 1. Frames were selected for superimposition which showed the endocardial mounds separated from each other as they appear during systole when blood is being forced through the truncus, and as they appear when they close the truncus against back flow at the close of each ventricular systole.

The details of the action of the endocardial mounds of the truncus during one complete cardiac cycle are shown by the 12 tracings of figure 3. These tracings were made from successive frames of film taken at the usual speed for silent moving pictures so that they represent the changes that have occurred at intervals of $1/16$ of a second. The dilation of the part of the ventricular lumen just proximal to the point of closure shown in the projections of frames 6 to 10 is interesting. It indicates the building up of pressure by a new contraction wave which has started at the sino-atrial end of the heart (Patten, B. M. and Kramer, T. C., 1933) and is "charging" the ventricle with blood. As this contraction wave advances the blood in the ventricle will force apart the truncus mounds (frames 11 and 12, and 1 to 3), and spurt out into the aortic roots. When the contraction wave reaches the truncus the lumen is closed, and the cardiac jelly tends for a brief time (frames 5 to 9) to be heaped up between the back pressure from the aortic system and the mounting pressure due to the recharging of the ventricle as a new cardiac cycle is initiated at the sino-atrial end of the heart. The effectiveness of these opposing pressures in heaping up the mounds is further indicated by the way the mounds flatten when the truncus is open, transmitting blood (frames 11 and 12 and 1 to 3). The rate of progression of the contraction wave at this stage of development is such that the atrioventricular mounds are closed as the ventricle is forcing blood out of the truncus, and the truncus mounds are closed as the atrioventricular mounds open to let a new charge of blood into the ventricle for the next cardiac cycle. The reciprocal action of the endocardial mounds at these two points

in the cardiac tube thus provides a simple but surprisingly smoothly operating valvular mechanism.

Perhaps more interesting than the valvular action itself are some of the possible theoretical implications involved. It is well known that an excised chick heart kept alive by tissue culture methods spends its energies in beating and, to



Fig. 3 Projection tracings of successive frames in a film analyzing closure at the cono-truncal region diagrammed in figure 2. The exposures were made at the rate of 16 per second and the 12 frames shown cover one complete cardiac cycle, from open phase to open phase.

a less extent, in unorganized growth of its tissue elements. It does not, as do many other explanted parts of the growing body, differentiate morphologically under the influence of its "inherent developmental potencies." This would naturally lead one to turn inquiringly toward the possible moulding influences exerted by the hydrodynamic effects of blood currents and eddies. The most thoughtful approach to the moulding effects on the chick heart which might possibly be exerted by the blood pouring through it has been made by Bremer ('28, '32a). If, as Bremer contends, the blood passes through the bent tubular heart as spiral streams which maintain their identity sufficiently to have an internal moulding effect, the cardiac jelly may be far more important in the differentiation of the heart than anyone has suspected. Its consistency appears to be such that it would be far more readily moulded by blood currents than would any of the organized tissues. Should this prove to be the case, the form taken, and the distribution acquired, by the cardiac jelly could well set the pattern followed by endocardial cushion tissue when it takes over, by cellular invasion, the areas which were occupied by the cardiac jelly of younger embryos. Thus it is possible that the pattern of the endocardial ridges and mounds that are so important in the formation of the septa and valves of the developing heart might be dependent on the previous hydrodynamic moulding effect of blood currents on a non-cellular gelatinous material.

Furthermore, it seems probable that the same sort of material that we call cardiac jelly when we encounter it between the endothelium and the myocardial sleeve of the tubular heart is of general occurrence in the spaces between the primary germ layers of young embryos. In sections one can trace the same sort of coagulated meshwork from the endocardial spaces, out between the two layers of the dorsal mesocardium and into the space between the pharynx and the ectoderm. Furthermore, a watery body fluid if under appreciable pressure would stretch out the superficial ectoderm into formless rotundity, or if under low pressure would permit

it to sag limply against underlying structures. A gelatinous material like cardiac jelly between ectoderm and entoderm would account for the way young embryos retain their characteristic shape when they are removed, and even when they are transected. It would also furnish a medium of such a consistency that mesenchymal cells might migrate in it instead of being limited in their excursions to amoeboid movement along a formed substratum such as the outer wall of the neural tube or the inner surface of the superficial ectoderm. The more one considers the places one finds mesenchymal cells in the growing embryo, the more inevitable it seems that there must be a material of just such properties through which they move. Such a conception is certainly an intriguing challenge to further experimental work.

LITERATURE CITED

- BREMER, J. L. 1928 An interpretation of the development of the heart. *Am. J. Anat.*, 42: Part I, 307-337.
- 1932a The presence and influence of two spiral streams in the heart of the chick embryo. *Am. J. Anat.*, 49: 409-440.
- 1932b Circulatory disturbances in operated chick embryos: Reversal of heart beat. *Anat. Rec.*, 51: 275-284.
- CHANG, C. 1932 On the reaction of the endocardium to the blood stream in the embryonic heart, with special reference to the endocardial thickenings in the atrioventricular canal and the bulbus cordis. *Anat. Rec.*, 51: 253-265.
- DAVIS, C. L. 1924 The cardiac jelly of the chick embryo. *Anat. Rec.*, 27: 201-202.
- 1927 Development of the human heart from its first appearance to the stage found in embryos of 20 paired somites. *Carnegie Cont. to Emb.*, 19: 245-284.
- FISCHEL, A. 1929 *Lehrbuch der Entwicklung des Menschen*. Julius Springer, Berlin, viii & 822 pp.
- PATTEN, B. M. 1922 The formation of the cardiac loop in the chick. *Am. J. Anat.*, 30: 373-397.
- PATTEN, B. M., AND KRAMER, T. C. 1933 The initiation of contraction in the embryonic chick heart. *Am. J. Anat.*, 53: 349-375.

PLATE

PLATE 1

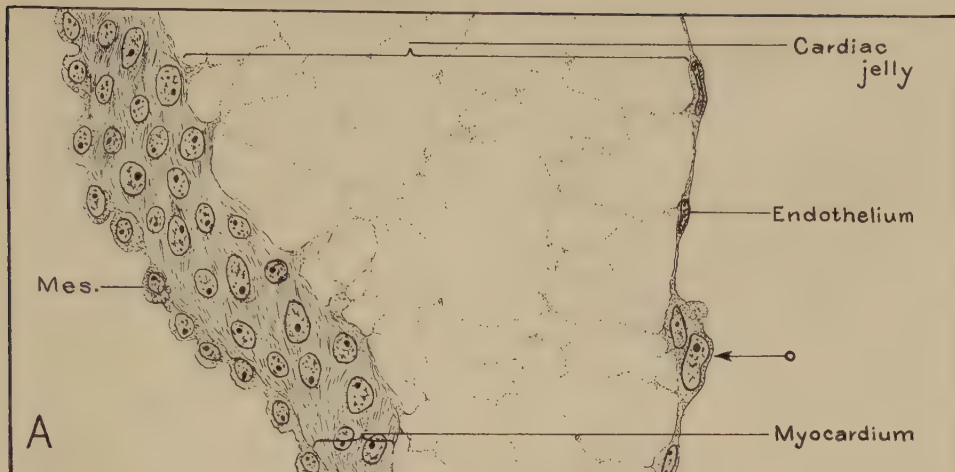
EXPLANATION OF FIGURES

Drawings showing stages in the cellular invasion of cardiac jelly with the resultant formation of endocardial cushion tissue. All the drawings were made from comparable areas in the cono-truncus to the same scale ($\times 1000$ original, reproduced $\times 750$).

A. From a 14-somite (± 36 hours) chick showing the primary non-cellular character of the cardiac jelly. Note the thinness of endothelium except for the place indicated by the arrow.

B. From a 33-somite (± 65 hours) chick showing the beginning of cellular invasion. Note the thickened character of the endothelium and the fact that cells have not as yet reached the myocardium.

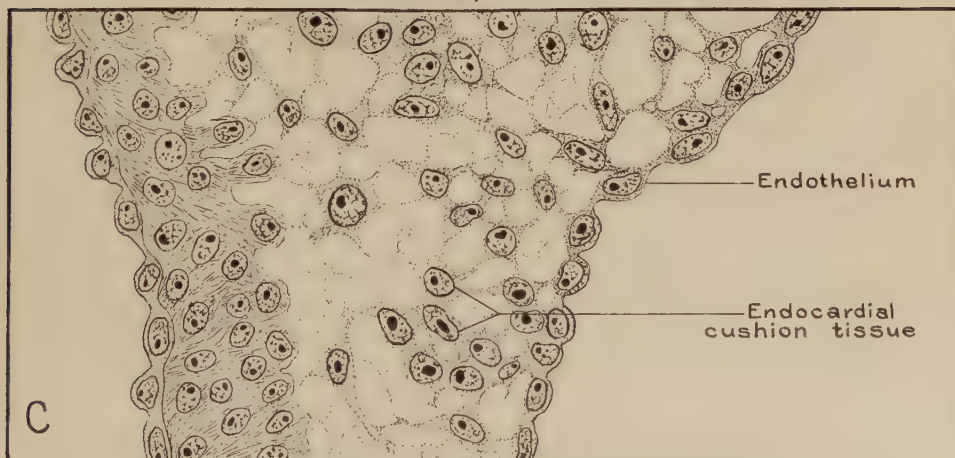
C. From a 40-41-somite chick (± 84 hours) showing cells throughout the territory formerly occupied by the cardiac jelly alone.



14 Somites, about 36 hrs.



33 Somites, about 65 hrs.



40-41 Somites, about 84 hrs.

GROWTH INHIBITION IN THE SKIN INDUCED BY PARENTERAL ADMINISTRATION OF ADRENOCORTICOTROPIN

BURTON L. BAKER, DWIGHT J. INGLE, CHOH H. LI AND
HERBERT M. EVANS

*Department of Anatomy, University of Michigan Medical School, Ann Arbor;
Research Laboratories, The Upjohn Company, Kalamazoo, Michigan
and Institute of Experimental Biology, University of
California, Berkeley*

SEVENTEEN FIGURES

For a number of years the anterior hypophysis has been known to play an important rôle in the regulation of body growth. In general, the effects of hypophyseal secretions on growth have been found to be of a stimulative character. The growth stimulating or inhibiting action of such hormones is demonstrated most precisely by the changes they elicit in those tissues which normally undergo the most rapid cell division. Hair is such a rapidly growing structure and its growth has been shown to be affected by the anterior hypophysis. For example, Smith ('30) demonstrated in the rat that pituitary activity is essential to the normal maturation of hair from the infantile to the adult type. Apparently the growth hormone is primarily responsible for this effect (Thompson and Gaiser, '32). Microscopically, hypophysectomy of young rats has been found to cause a general atrophy of the skin with reduction in size of connective tissue fibers, hair follicles and their papillae (Snow and Whitehead, '35). These changes were reversed by treatment of hypophysectomized rats with extracts containing the growth hormone. The general conclusion to be drawn from these early experiments

is that the growth hormone stimulates the hair to grow and mature.

The discovery that growth of hair is accelerated by adrenalectomy (Butcher and Richards, '39; Butcher, '41; Ralli and Graef, '43) and that this rapid growth after adrenalectomy may be inhibited by the administration of cortical preparations indicates that the adrenal cortex normally acts either directly or indirectly as an inhibitor of hair growth. Since the secretory activity of at least part of the adrenal cortex is controlled by the anterior hypophysis, it is pertinent to investigate the manner in which the anterior hypophysis affects the growth of hair when it acts through the adrenal cortex. This problem is unusually challenging in view of the evidence just cited which indicates that the adrenal cortex inhibits hair growth. Is it possible that the anterior hypophysis possesses the capacity for both stimulating and inhibiting hair growth—stimulating it by direct action of the growth hormone and inhibiting it by accelerating the release of cortical steroids? Might it have some influence also on other cutaneous structures which exhibit continuous or intermittent cell division such as the epidermis and sebaceous glands? The development of a method for preparation of hypophyseal adrenocorticotrophic hormone in a comparatively pure form by Li, Evans and Simpson ('43) makes possible an investigation of the action of the anterior hypophysis on the skin when it acts by way of first stimulating the adrenal cortex. This study, in which Doctor Li's preparation has been used attempts to answer the questions which have been raised.

MATERIALS AND METHODS

The experimental procedures employed in this study have been described in detail elsewhere (Baker, Ingle, Li and Evans, '48). In summary, adult male rats of the Sprague-Dawley strain were force-fed diets high in carbohydrate, fat or protein. Following a period of adaptation to this type of feeding, they were injected with adrenocorticotropin. In experiment 1, one rat on each of the three diets received 1 mg of adrenocortico-

tropin per day for 21 days, with the same number of animals serving as controls. Experiment 2 was similar except that the dosage of hormone was increased to 3 mg per day. In experiment 3, 8 mg of adrenocorticotropin were injected daily for 10 days into two rats on the high fat and high protein diets with two controls on each diet; on the high carbohydrate diet, 4 animals were injected with the hormone and there were two controls. The amount of adrenocorticotropin administered daily was divided into 8 doses which were injected at intervals of two hours. The control animals were given injections of 0.9% sodium chloride at the same times.

At autopsy samples of skin measuring about 1×0.5 cm were removed from the dorsum of the body in the thoracic region. This method of sampling did not take into account the waves and cycles of hair growth which occur in the rat (Butcher, '34). Therefore, in order to obviate the possibility that the skins of treated rats which showed no hair growth might have been taken from normally inactive areas and those of the controls from normally active areas, experiment 4 was carried out with three rats receiving 3 mg of adrenocorticotropin daily and three others serving as controls. This procedure duplicated the conditions of experiment 2, except that all of these rats were fed a medium carbohydrate diet (Ingle, Nezamis and Prestrud, '47). Since it has been reported by Butcher ('34) that hair in rats grows in waves which begin mid-ventrally and proceed dorsally, a strip of skin 4 mm wide and extending from the mid-ventral to mid-dorsal line was excised from the lumbar region of each rat at autopsy. It was sectioned in such a way that a complete microscopic picture of the skin was thus available for one-half the circumference of the body.

The skin samples were fixed in Bouin's fluid and imbedded in paraffin. In the case of the samples removed from the dorsum of the back, approximately 2.6 to 3 mm of skin in the paraffin blocks was sectioned serially and studied. The stains used were Harris' hematoxylin and eosin, Regaud's iron hematoxylin and Masson and the Weigert procedure for

elastic fibers. In experiment 4, small pieces of abdominal skin were fixed in chilled 80% alcohol and prepared for the study of alkaline phosphatase by the method of Gomori ('39); other pieces were fixed in 4% basic lead acetate and stained metachromatically with toluidine blue for demonstration of mast cells.

In the earlier experiments no attempt was made to study the re-growth of hair on shaved areas during treatment. However, in experiment 4 the hair was clipped from the entire body at the beginning of the experiment. Then, at weekly intervals during treatment, records were made of the extent of hair growth during the preceding week by sketching the growth pattern and the hair was clipped again.

OBSERVATIONS

Comparison of figures 1 and 3 with figures 2 and 4 demonstrates that adrenocorticotropin caused an apparent thinning of the skin. However, closer scrutiny reveals that this appearance resulted largely from the reduction in thickness of the subcutaneous layer of loose fibro-elastic connective tissue and fat (*panniculus adiposus*), a change so marked as to be readily discernible upon macroscopic inspection of the stained slide. In general, the *panniculus adiposus* was obliterated completely in which case a dense mass of collagenous fibers approximated directly the *panniculus carnosus* muscle. In other rats the reduction was not as great and narrowed patches of fat and loose connective tissue remained in some localities especially around whatever hair follicles might still be present in this region (fig. 4). The effect seemed to be greatest following the 3 mg daily dose administered for 21 days and less when 1 mg was given daily for this period or 8 mg for the shorter period of 10 days.

Microscopic measurement of the dermis demonstrated that the thickness of this layer of the skin proper was not altered significantly by either diet or treatment with the hormone. It should be mentioned in this connection that accurate measure-

ment was difficult because of the irregularity of the boundary between the dermis and panniculus adiposus.

Epidermis. Dorsally in the control animals a definite layering of the epidermis was evident with stratum germinativum, corneum and, at least, a discontinuous stratum granulosum being present constantly. Occasionally a deeply eosinophilic layer could be seen external to the stratum granulosum which resembled somewhat the stratum lucidum of human skin. The stratum germinativum and, in places, the stratum granulosum were several cell layers in thickness (fig. 5). The epidermis became progressively thinner with a less prominent stratum granulosum toward the ventral side of the body.

Treatment with adrenocorticotropin caused a striking change in the surface contour of the skin. Whereas in the control rats this surface was rather even and smooth especially in the dorsal area (figs. 1 and 3) following treatment the epidermis was thrown up into papilla-like folds giving it a "crinkly" appearance on microscopic observation (figs. 2, 8). This alteration was accompanied by a thinning of the epidermis which was most striking dorsally because in this region the epidermis normally attains its greatest thickness. In some locations the cellular part was reduced to one or two layers in thickness with considerable flattening of the cells (figs. 5, 6). The greatest change seemed to have occurred in the stratum germinativum and stratum granulosum. The atrophy of the former layer could have been incident to an inhibition of cellular proliferation. Particularly outstanding was the great reduction in size and number of the keratohyalin granules in the stratum granulosum (fig. 6). Assuming these granules to represent one stage in the formation of keratin, this alteration might be interpreted as being indicative of a defect in the process of cornification. Indeed, in some rats which were affected by the treatment more markedly, the atrophic epidermis possessed a comparatively thin stratum corneum (fig. 6). However, the observer could not always ascertain how much the stratum corneum had been altered by the experimental conditions because of the impossibility of assessing the extent

to which the layer had been disturbed by mechanical attrition. In such atrophic epidermis no eosinophilic layer which might be regarded as a stratum lucidum was present. All of these changes were found consistently on the 3 mg dose except in one of the 6 treated rats in which the epidermis was not significantly thinner than that of its control. These effects occurred variably on the 1 and 3 mg dosage.

Hair. The full magnitude of the extent to which adrenocorticotropin inhibited growth of hair could not be appreciated from the small pieces of skin which were removed from the back in the first experiments. The reliability of comparisons made by this procedure between the rate and extent of hair growth in a control and an experimental rat is dependent upon the expectation that the pattern of hair growth, except for the hormone treatment, would have been identical in both animals. One could not validly make such an assumption, especially since the control and experimental animals in our studies were not litter mates. Nevertheless, in these specimens suggestive evidence of retarded development of hair follicles and bulbs due to treatment with adrenocorticotropin was obtained.

This repression seemed to be related directly to the dosage and duration of treatment. On the 1 mg dose for 21 days, there was evidence of inhibition following treatment with the hormone in only the animal on the carbohydrate diet in which case no follicles were found to extend deeper than the middle of the dermis in the serial sections which were studied. With increase of the dosage to 8 mg for the shorter period of 10 days, inhibition of hair growth became more definite. In one adrenocorticotropin-protein treated rat there was no effect. However, in all of the other rats, there were fewer follicles which reached to the panniculus adiposus and, in general, the greater proportion of those which did were atrophic (figs. 3 and 4). This stood in contrast to the controls in which more of the hairs possessed large growing bulbs.

Increasing the daily dose to 3 mg for the longer period of 21 days resulted in still more impressive evidence of inhibition

of hair growth. In the serial sections of the skin of the three animals in experiment 2 treated at this dosage, in only one rat (carbohydrate diet) did a rare follicle reach the panniculus adiposus and the few which did, possessed bulbs far smaller than those in the controls. In the other two treated rats, no follicles were found to extend beyond the middle of the corium (fig. 2). In contrast, in all of the controls numerous follicles extended to the panniculus adiposus and many of these possessed large bulbs (fig. 1).

In experiment 4 on the 3-mg daily dosage any doubt as to the marked inhibitory effect of adrenocorticotropin on hair growth was removed. Re-growth of hair was observed grossly on these animals. Treatment with adrenocorticotropin had no apparent effect during the first two weeks of the experiment. However, at the end of three weeks there was intense inhibition of hair growth in the treated rats as compared with the controls (figs. 13 and 14). During the third week, hair growth had occurred in the latter animals over almost the entire thoracico-lumbar region. In contrast, only thin, sparse hair had grown in small areas of the skin of each of the three adrenocorticotropin-treated rats. Macroscopic observation indicated that variation of the diet did not modify the inhibitory action of adrenocorticotropin on the growth of hair.

Microscopic study of a strip of skin from one-half the circumference of the body in these rats offered confirmatory evidence of the markedly reduced rate of hair growth. In the three controls, hair was growing throughout most of the strip as shown by the presence of many large active bulbs in the panniculus adiposus. This microscopic picture confirmed the previously described gross observations which were made on these animals. In contrast, two of the three treated rats were almost completely inactive with only an occasional hair bulb being found and most of these were small and not surrounded by fat. In the third treated rat, the amount of inhibition was still very great but in this animal, hair was growing through about one-twentieth or less of the strip of skin. In its control, growing bulbs were found almost throughout the entire strip.

In samples of skin taken from rats treated with adrenocorticotropin, epithelial buds presaging a period of active hair growth could be found at the end of some of the dormant hair follicles. These clusters of cells, however, appeared to be in an atrophic condition since the cells were small and nuclei densely packed (fig. 15). Additional evidence of inactivity is found in the absence of alkaline phosphatase in or around such cells (fig. 17). Johnson, Butcher and Bevelander ('45) demonstrated small amounts of this enzyme in the epithelial hair buds and large amounts in the surrounding connective tissue of growing hair in newborn rats. In our control specimens, growing epithelial buds showed intense phosphatase activity as did the surrounding connective tissue. As the epithelial buds expanded into large hair-forming bulbs the enzyme was most concentrated in the connective tissue papilla and sheath of the follicle and, frequently, in the periphery of the epithelial part of the bulb (fig. 16).

Sebaceous glands. Since sebaceous glands are of a holocrine type, their epithelial cells proliferate continuously in order to replace those which are converted into and secreted as droplets of oil. It might be expected that a growth-inhibiting substance such as adrenocorticotropin would inhibit this proliferation and, thus, cause an atrophy of the gland. Some difficulty is attendant upon the determination of such effects since in any histological section of skin, the sebaceous glands normally vary in size and distribution. Therefore, only extreme changes are detected easily. It did seem clear that on the 1 mg dosage no effect could be discerned in the size or number of these structures. However, in the small dorsal skin samples from rats on the 3- and 8-mg dosages, there was in all cases a variable reduction in size of the glands but it was not possible to be certain of a significant reduction in number although this was indicated in a few cases. Comparison of figures 7 and 8 illustrates the maximum evidence of sebaceous gland atrophy which was obtained by such a sampling method. Study of the long strips of skin from the rats in experiment 4 indicated that the effect of adrenocortico-

tropin on the sebaceous glands was not as great as was deduced from the earlier studies. In the treated animals one could find some glands of an approximately normal size even though, in general, they were probably somewhat smaller. Therefore, it is to be concluded that under the conditions of these experiments, the atrophy of the sebaceous glands induced by adrenocorticotropin did not compare with the changes induced in the epidermis or hair. The observation that considerable alkaline phosphatase activity was still evident in these glands after treatment with the hormone (fig. 17) further suggests that their function had not been suppressed greatly.

Dermis. Adrenocorticotropin caused a variable increase in compactness of arrangement of the constituent collagenous fibers of the dermis which in most animals was very striking. This change seemed to be accompanied by a swelling of the collagenous fibers with a parallel reduction in the intervening tissue spaces (figs. 11 and 12). Mast cells as revealed by toluidine blue, were still present after treatment with adrenocorticotropin but were probably reduced somewhat in number.

Elastic fibers in the control animals were most numerous in the upper one-third to one-half of the corium, around hair follicles and sebaceous glands and beneath the panniculus carnosus muscle. There was no significant change induced in them by adrenocorticotropin (figs. 9 and 10). However, in some treated rats the fibers may have been slightly more coarse as well as more concentrated around the hair follicles. The latter effect probably was incident to the atrophy of the hair follicles.

DISCUSSION

In an investigation of the relationship of the hypophysis and adrenal cortex to the regulation of growth, it is significant that in these experiments hypophyseal adrenocorticotropin caused a reduction in size of the epithelial integumentary structures which exhibit more or less continuous cellular division, this atrophy being demonstrated by the epidermis, the

growing parts of hair and less significantly by the sebaceous glands. These observations extend the earlier ones of Moon ('37) who reported a greatly retarded growth of hair over the shaved areas of immature gonadectomized rats injected with large doses of crude adrenocorticotrophic extract. It would appear that these findings fit into the general pattern of growth inhibition which apparently follows treatment with adrenocorticotropin. All of the animals in this experiment which received injections of the hormone lost weight except those on the protein diet. Nevertheless, these latter rats gained less weight than their controls (Ingle, Prestrud, Li and Evans, '47). Adrenocorticotropin has been shown to inhibit body growth (Evans, Simpson and Li, '43), proliferation of the epiphyseal cartilage (Becks, Simpson, Li and Evans, '44) and to antagonize the action of the growth hormone (Marx, Simpson, Li and Evans, '43; Becks, Simpson, Marx, Li and Evans, '44). Marx et al. ('43) showed that diminished food consumption did not explain the growth inhibition caused by adrenocorticotropin. This factor was eliminated in our experiment by force-feeding the animals. It is also possible that suppressed cellular proliferation may be a factor in the profound atrophy of the thymus which we found to follow treatment with adrenocorticotropin.

In the studies of Evans and his collaborators just cited, inhibition of growth did not ensue following treatment of adrenalectomized animals with adrenocorticotropin which demonstrates that this effect is mediated by the adrenal glands. It may be presumed that in our experiments the atrophy of the epidermis and its derivatives was brought about in a similar manner. Furthermore, urinalyses in these animals (Ingle, Prestrud, Li and Evans, '47) gives suggestive evidence as to the specific steroids whose release by the adrenal cortex may be stimulated by adrenocorticotropin. All of the rats treated with adrenocorticotropin showed an increased non-protein nitrogen excretion and some of those concurrently fed high and medium carbohydrate diets exhibited glycosuria. Such effects are characteristic actions of the 11-

oxysteroids. Thus, it appears possible that adrenocorticotropin caused the secretion by the adrenal cortex of C-11 oxygenated steroids which in turn inhibited growth by promoting the catabolism of protein. Evidence for the inhibition of growth by these steroids will be summarized subsequently (Baker and Whitaker, this journal).

The changes brought about in the connective tissue of the dermis by adrenocorticotropin raise an important question as to how this effect may be involved in the inhibition of growth of the epithelial structures in the skin. Is it possible that the primary effect of adrenocorticotropin is on the connective tissue and that the epithelial atrophy is secondary? One might expect that structural modification of the dermis could have impaired the nutrition of the epidermis, hair and sebaceous glands either by compression of the small blood vessels or by interfering with the functioning of the tissue fluid. A satisfactory evaluation of the state of the vascular bed by microscopic study of our preparations proved to be impossible. Apparent swelling of collagenous fibers is suggestive of a disturbance in the electrolytes of the tissue fluids. Ingle, Prestrud, Li and Evans ('47) observed an increased excretion of potassium in these rats to be induced by adrenocorticotropin. Some loss of sodium and chloride occurred during the first two days of treatment but this effect was not regarded as significant. At the present time one cannot evaluate the proper place of connective tissue in the chain of events leading to suppression of growth by adrenocorticotropin.

The atrophy of the panniculus adiposus is significant in view of the fatty infiltration of the liver which occurred in those rats which were treated with adrenocorticotropin and fed a high carbohydrate diet (Baker et al., '48). In this report two possible explanations were offered for the accumulation of fat in the liver: (1) interference with carbohydrate metabolism in the liver with conversion of the carbohydrate to fat and deposition of it in the liver and (2) interference with the transport of fat between the body depots and the liver. If the latter explanation is a factor in the accumulation

of fat in the liver after treatment with adrenocorticotropin, then the marked atrophy of this extensive depot (panniculus adiposus) becomes of utmost importance.

The presence or absence of a well-developed panniculus adiposus must bear some relation to the growth status of hair. However, the exact nature of this relationship has not been clarified. Butcher ('34) concluded that the presence of a well-developed fatty layer is not prerequisite to the growth of hair. Nevertheless, he observed the resting condition of the hair to be followed by a reduction in fatty content of the subcutaneous layer. It was his opinion that the presence of fat facilitated more rapid growth of hair.

Finally, it seems that some of the characteristics of skin after treatment with adrenocorticotropin are similar to those of normal skin which is in a state of inactive hair growth. The reduction in the panniculus adiposus and the undeveloped state of the hair follicles fits into this category. However, the increased compactness of the dermis and atrophy of the epidermis appeared to be more extreme modifications than occur in the skin concurrently with the cessation of hair growth. Our procedures did not permit an analysis of the true nature of the connective tissue changes.

SUMMARY

Adult male rats were force-fed high carbohydrate, high fat, high protein or medium carbohydrate diets and treated with adrenocorticotropin at daily dose levels of 1 or 3 mg for 21 days and 8 mg for 10 days. As a result of treatment with this hormone, there occurred thinning of the epidermis, reduced growth of hair, an inconsistent reduction in size of the sebaceous glands, increased compactness of the dermal connective tissue and reduction of the panniculus adiposus. Dosage and duration of treatment seemed to be important factors in eliciting these responses, the most marked effect being obtained with the 3-mg daily dose administered for 21 days. Variation of the diet did not modify the response to the hormone.

LITERATURE CITED

- BAKER, B. L., D. J. INGLE, C. H. LI AND H. M. EVANS 1948 The effect on liver structure of treatment with adrenocorticotropin under varied dietary conditions. *Am. J. Anat.*, *82*: 75-103.
- BECKS, H., M. E. SIMPSON, C. H. LI AND H. M. EVANS 1944 Effects of adrenocorticotrophic hormone (ACTH) on the osseous system in normal rats. *Endocrin.*, *34*: 305-310.
- BECKS, H., M. E. SIMPSON, W. MARX, C. H. LI AND H. M. EVANS 1944 Antagonism of pituitary adrenocorticotrophic hormone (ACTH) to the action of growth hormone on the osseous system of hypophysectomized rats. *Endocrin.*, *34*: 311-316.
- BUTCHER, E. O. 1934 The hair cycles in the albino rat. *Anat. Rec.*, *61*: 5-20.
- 1941 Accelerated hair growth in the rat after adrenalectomy cannot be attributed to the thyroid. *Proc. Soc. Exp. Biol. and Med.*, *48*: 120-122.
- BUTCHER, E. O., AND R. A. RICHARDS 1939 The relation of the adrenals to the retarded hair growth in underfed albino rats. *Endocrin.*, *25*: 787-792.
- EVANS, H. M., M. E. SIMPSON AND C. H. LI 1943 Inhibiting effect of adrenocorticotrophic hormone on the growth of male rats. *Endocrin.*, *33*: 237-238.
- GOMORI, G. 1939 Microtechnical demonstration of phosphatase in tissue sections. *Proc. Soc. Exp. Biol. and Med.*, *42*: 23-26.
- INGLE, D. J., J. E. NEZAMIS AND M. C. PRESTRUD 1947 The effect of diethylstilbestrol upon alloxan diabetes in the male rat. *Endocrin.*, *41*: 207-212.
- INGLE, D. J., M. C. PRESTRUD, C. H. LI AND H. M. EVANS 1947 The relationship of diet to the effect of adrenocorticotrophic hormone upon urinary nitrogen, glucose and electrolytes. *Endocrin.*, *41*: 170-176.
- JOHNSON, P. L., E. O. BUTCHER AND G. BEVELANDER 1945 The distribution of alkaline phosphatase in the cyclic growth of the rat hair follicle. *Anat. Rec.*, *93*: 355-361.
- LI, C. H., H. M. EVANS AND M. E. SIMPSON 1943 Adrenocorticotrophic hormone. *J. Biol. Chem.*, *149*: 413-424.
- MARX, W., M. E. SIMPSON, C. H. LI AND H. M. EVANS 1943 Antagonism of pituitary adrenocorticotrophic hormone to growth hormone in hypophysectomized rats. *Endocrin.*, *33*: 102-105.
- MOON, H. D. 1937 Inhibition of somatic growth in castrate rats with pituitary extracts. *Proc. Soc. Exp. Biol. and Med.*, *37*: 34-36.
- RALLI, E. P., AND I. GRAEF 1943 Stimulating effect of adrenalectomy on hair growth and melanin deposition in black rats fed diets adequate and deficient in the filtrate factors of vitamin B. *Endocrin.*, *32*: 1-12.
- SMITH, P. E. 1930 Hypophysectomy and a replacement therapy in the rat. *Am. J. Anat.*, *45*: 205-274.
- SNOW, J. S., AND R. W. WHITEHEAD 1935 Relationship of the hypophysis to hair growth in the albino rat. *Endocrin.*, *19*: 88-96.
- THOMPSON, K. W., AND D. W. GAISER 1932 The effect of diet and pituitary growth-hormone on hypophysectomized rats. *Yale J. Biol. and Med.*, *4*: 677-690.

PLATE 1

EXPLANATION OF FIGURES

The specimens illustrated in figures 1-4 were fixed in Bouin's fluid, sectioned at $10\ \mu$, and stained with Harris' hematoxylin and eosin. Panniculus carnosus muscle is at the left. Figures 1-12 are of skin samples removed from the back. $\times 28$.

1 Rat 1401, fat control. The thick epidermis possesses a rather regular surface. Many large, growing hair bulbs reach the thick panniculus adiposus. $\times 28$.

2 Rat 1301, fat, 3 mg adrenocorticotropin per day for 21 days. The surface is "crinkly" and the epidermis is thinner and of more variable thickness. No hair follicles reach below the upper one-third of the dermis. The dermis is more dense and the panniculus adiposus is greatly reduced. $\times 28$.

3 Rat 1201, carbohydrate control. The characteristics are similar to those illustrated in figure 1. $\times 28$.

4 Rat 1404, carbohydrate, 8 mg adrenocorticotropin per day for 10 days. The effects are similar to those shown in figure 2 except that they are less marked. $\times 28$.

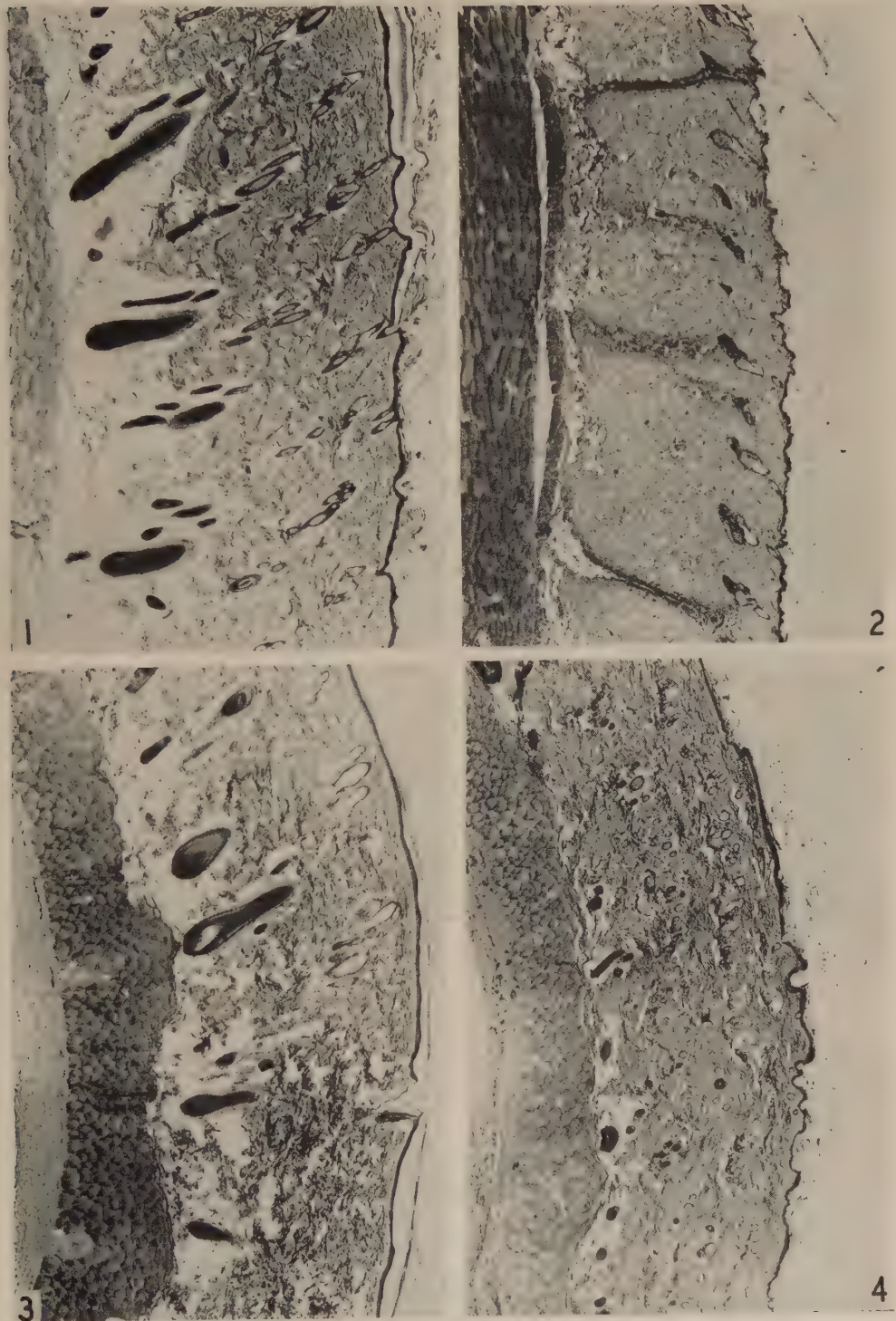


PLATE 2

EXPLANATION OF FIGURES

5 Rat 1204, carbohydrate control. The epidermis is thick with stratum germinativum, granulosum and corneum showing distinctly. An indistinct stratum lucidum is visible above the stratum granulosum. The keratohyalin granules (black) of the latter layer are quite prominent. 6 μ . Hematoxylin and eosin. $\times 464$.

6 Rat 1304, carbohydrate, 8 mg adrenocorticotropin per day for 10 days. All strata of the epidermis are atrophic. Only a few small keratohyalin granules are present in the stratum granulosum. Technique as in figure 5.

7 Rat 1401, fat control. The epidermis is thick and smooth. Sebaceous glands are large. 10 μ . Hematoxylin and eosin. $\times 52$.

8 Rat 1301, fat, 3 mg adrenocorticotropin per day for 21 days. Surface is "crinkly" and the epidermis is generally thinner and of variable thickness. Sebaceous glands are atrophic. Collagenous fibers of the dermis are swollen. Technique as in figure 7.

9 Rat 701, protein control. The black lines in the dermis are elastic fibers. Weigert's resorcin fuchsin with no counterstain. $\times 112$.

10 Rat 801, protein, 1 mg adrenocorticotropin per day for 21 days. There is no significant change in the elastic fibers. Technique as in figure 9.

11 Rat 1401, fat control. Sharply defined collagenous fibers are illustrated in the dermis with definite tissue spaces between them. A few of these spaces are widened further by technical distortion. 10 μ . Hematoxylin and eosin. $\times 140$.

12 Rat 1301, fat, 3 mg adrenocorticotropin per day for 21 days. This area was selected from the same depth in the dermis as that shown in figure 11 and shows the increased compactness of the dermis with apparent swelling of collagenous fibers. Some of these spaces are also artefacts. Technique as in figure 11.

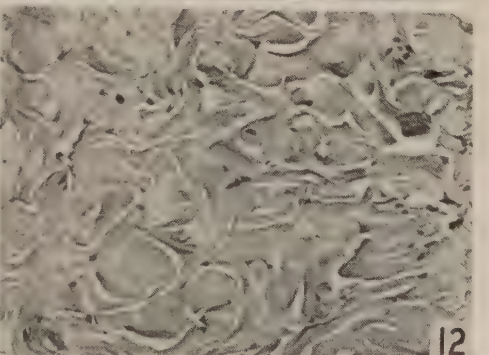
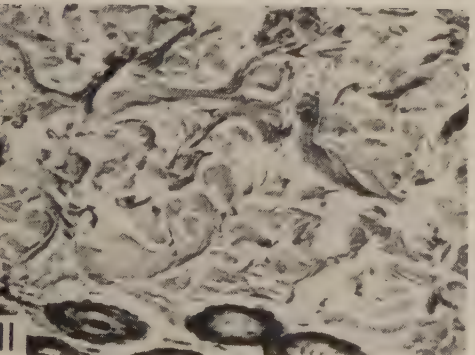
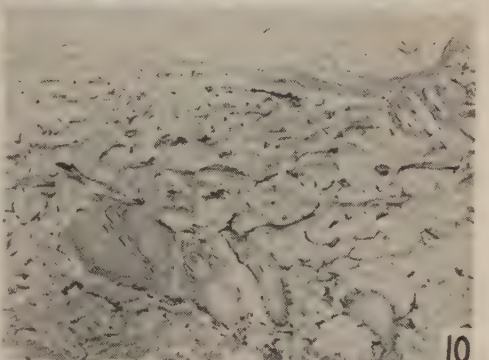
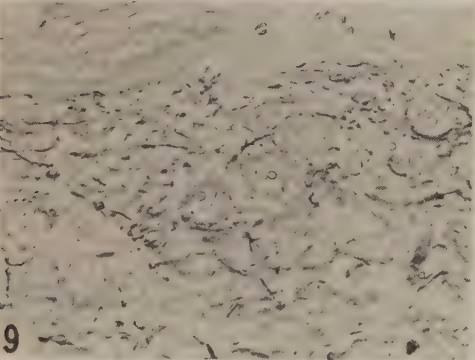
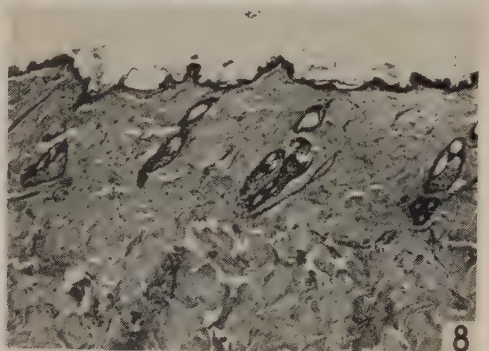
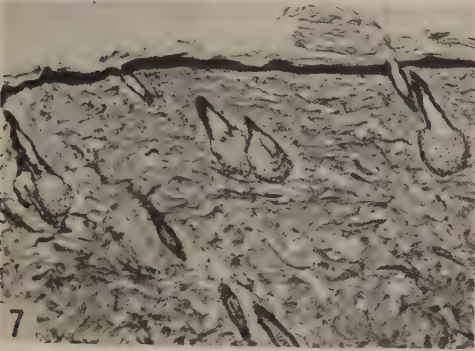
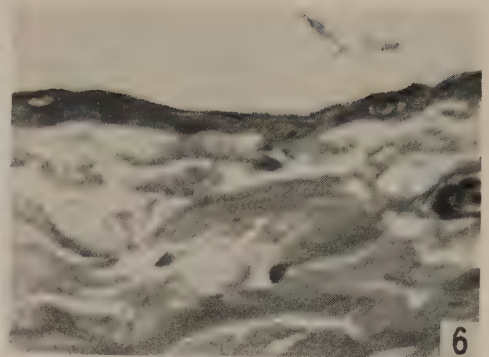
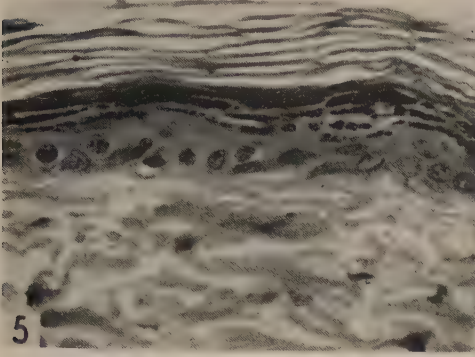


PLATE 3

EXPLANATION OF FIGURES

All illustrations on this plate are of rats fed a medium carbohydrate diet.

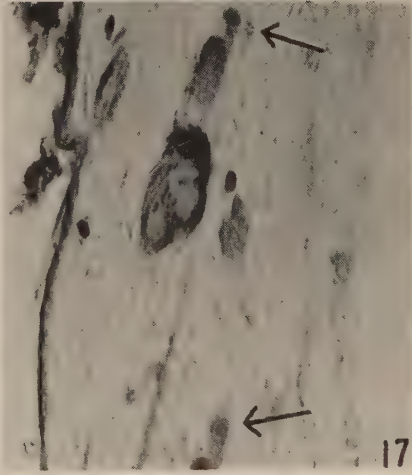
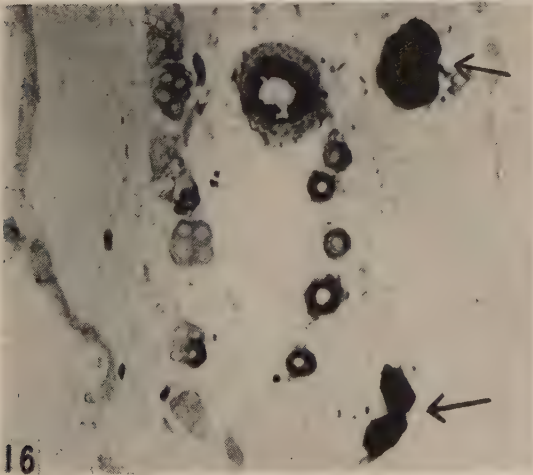
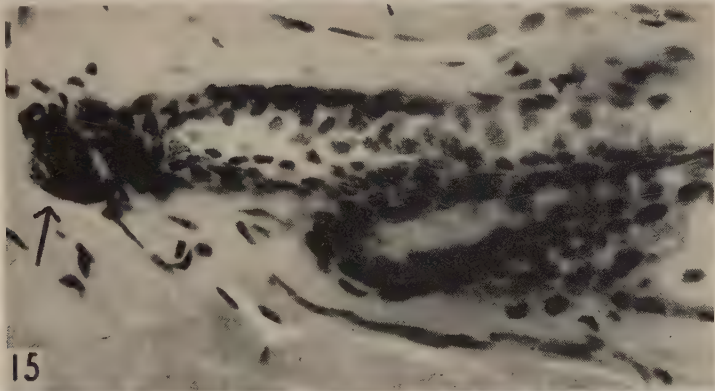
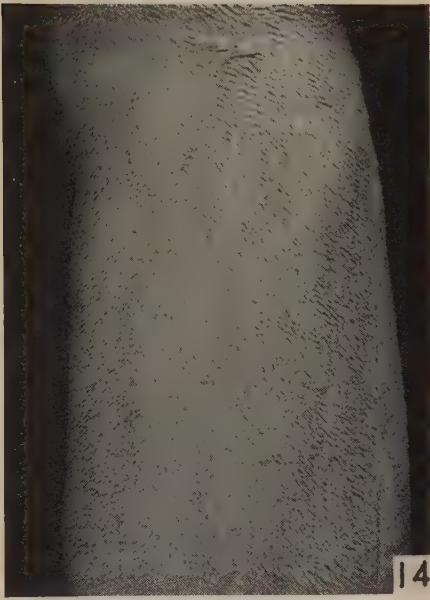
13 Rat 18003, control; photograph taken at the termination of the experiment. General growth of hair had occurred during the third week of the experiment.

14 Rat 17003, 3 mg of adrenocorticotropin daily for 21 days. Growth of hair had been suppressed almost completely during the third week of treatment.

15 Rat 15005, 3 mg of adrenocorticotropin daily for 21 days. The epithelial bud (arrow) is composed of small densely packed nuclei. This illustrates the stage in which growth of hair was arrested by the hormone. 10 μ . Hematoxylin and eosin. $\times 480$.

16 Rat 16004, control. The upper arrow points to a growing hair bulb with a central connective tissue papilla, both the papilla and epithelium giving an intensely positive reaction for alkaline phosphatase. The lower arrow points to two smaller hair buds which are likewise positive. 10 μ . Gomori alkaline phosphatase procedure, 24-hour incubation, pH 9.4, sodium barbital buffer. $\times 136$.

17 Rat 15005, 3 mg adrenocorticotropin daily for 21 days. Epithelial buds (arrows) are practically negative for alkaline phosphatase. The periphery of the sebaceous gland in the center is positive. Technique as for figure 16.



GROWTH INHIBITION IN THE SKIN FOLLOWING DIRECT APPLICATION OF ADRENAL COR- TICAL PREPARATIONS ¹

BURTON L. BAKER AND WAYNE L. WHITAKER

Department of Anatomy, University of Michigan, Ann Arbor

ELEVEN FIGURES

Examination of the skin of rodents following adrenalectomy has shown that the adrenal glands exert a regulatory influence over the growth of hair. Butcher and Richards ('39), Butcher ('41) and Ralli and Graef ('43) observed accelerated growth of hair after adrenal ablation which began as early as two to three days following the operation. Stimulation of hyperplasia of the hair follicles and bulbs by adrenalectomy was sufficiently strong to overcome the atrophy of the growing portion of hair which had been induced by under-feeding (Butcher and Richards, '39) or by prior feeding of a diet deficient in the filtrate factors of the vitamin B complex (Ralli and Graef, '45). Conversely, the latter authors found that adrenal cortical extract and, particularly, desoxycorticosterone acetate reduced the hyperplasia of hair follicles and bulbs which followed adrenalectomy. Thus, it appears that the adrenal cortex normally acts directly or indirectly as an inhibitor of hair growth.

Likewise, it has been shown that parenteral administration of a hormone of the anterior hypophysis (adrenocorticotropin), may inhibit growth of hair, epidermis and, to a lesser degree, of sebaceous glands (Baker, Ingle, Li and Evans, this journal). In that report other evidence was summarized

¹ Aided by a grant from the United States Public Health Service.

which suggests that adrenocorticotropin prepared by the method of Li, Evans and Simpson ('43) causes the accelerated release by the adrenal cortex of the 11-oxysteroids² (glucocorticogenic). Presumably, the growth inhibition which follows the injection of adrenocorticotropin is effected ultimately by these hormones. At least two basic questions are raised by this thesis. First, is this effect mediated by any other endocrine gland such as the hypophysis? Second, since the 11-oxysteroids are known to catabolize protein to form carbohydrate in the liver, is the liver an essential link in the growth-inhibiting action of the 11-oxysteroids?

In order to answer these questions, experiments have been carried out in which cortical steroids were applied directly to the end-organ (skin) and the growth of epithelial structures within it was studied. It was discovered that one 11-oxysteroid (11-dehydro 17-hydroxycorticosterone) and an aqueous adrenal extract stopped the re-growth of hair (Whitaker and Baker, '48). The present study deals primarily with the microscopic picture found in the skin of these rats and furnishes additional evidence that the 11-oxysteroids are responsible for the growth inhibition induced by adrenocorticotropin.

MATERIALS AND METHODS

The site chosen for study was the dorsal half of the neck since this area is relatively inaccessible to licking and scratching. The area studied was clipped weekly and photographic or sketched records made of the extent and location of hair growth during the preceding week. The hormones were applied just caudal to the right ear. Adult male rats (Long-Evans and Wistar strains) were divided into three groups and treated daily: (1) 13 rats received 0.1 cm³ of a 1 mg per cubic centimeter solution of 11-dehydro 17-hydroxycorticoster-

²By "11-oxysteroids" is meant those compounds isolated from the adrenal cortex which are oxygenated in the C-11 position and which, in general, affect protein and carbohydrate metabolism. These include corticosterone, 11-dehydrocorticosterone, 17-hydroxycorticosterone, and 11-dehydro 17-hydroxycorticosterone. In contrast, certain other compounds have no oxygen at the C-11 position, such as desoxycorticosterone, and these exert their main effect on electrolyte metabolism.

terone in 25% alcohol, (2) 8 rats received 0.1 cm³ of 25% alcohol and (3) 5 rats, 0.1 cm³ of aqueous cortical extract.³ Eleven-dehydro 17-hydroxycorticosterone is an "11-oxysteroid" and the cortical extract used contains low concentrations of this and other cortical steroids oxygenated at the C-11 position. Each animal was kept in an individual cage. The studies were carried out from January 24, 1948 to March 22, 1948 and during this period the varying hair growth patterns in the alcohol controls (group 2) and in groups 1 and 3 for the first three to 4 weeks of treatment were, with the exception of minor variations, bilaterally symmetrical. Therefore, the left side of the neck served as the animal's own control as well as group 2 serving as a control for groups 1 and 3.

Skin biopsies of the right and left sides of the neck in all animals were made at varying intervals from 21 to 47 days after the beginning of treatment. Skin was taken from the same relative areas on both sides. Some of the biopsies were made with a no. 6 Keyes skin punch while in other cases, a strip of skin 3 mm wide extending from the dorsal mid-line to the lateral line of the neck was excised from both sides. This report is based on 14 paired biopsies from group 1, 8 from group 2 and 6 from group 3.

The skin samples were fixed in either Zenker-formol, Zenker-base, Carnoy's or Bouin's fluid and stained with eosin and methylene blue (disodium phosphate-citric acid and sodium acetate-acetic acid buffers), hematoxylin and eosin, the Weigert procedure for elastic fibers, and the Masson technique.

OBSERVATIONS

As observed macroscopically, percutaneous application of 11-dehydro 17-hydroxycorticosterone stopped re-growth of hair and formation of associated pigment in the skin of the area treated as early as three weeks after the initiation of

³ We wish to express our appreciation to Dr. J. J. Pfiffner of Parke, Davis and Company, for supplying us with aqueous adrenal extract (Eschatin).

treatment. These processes were held in abeyance for the duration of the experiment. There was no disturbance in hair growth on the left side (non-treated area) in such animals, thus creating an asymmetry in the growth pattern. On the basis of the evidence at hand the 4 Wistar albino rats treated with 11-dehydro 17-hydroxycorticosterone did not show as complete an inhibition of hair growth at three weeks as did all of the 9 Long-Evans black-hooded rats. However, by the 4th or 5th week inhibition was equally as marked.

In group 2, treatment with the solvent, 25% alcohol, caused no significant disturbance in the growth pattern.

The cortical extract induced inhibition of hair growth which at 6 weeks was as extensive as that induced by 11-dehydro 17-hydroxycorticosterone at three weeks (fig. 11).

The microscopic picture of the skin was similar after treatment with 11-dehydro 17-hydroxycorticosterone and the aqueous extract except that in the latter instance a longer period of treatment was required to elicit the effects. In all cases changes observed in the treated areas are considered in relationship to the left or normal non-treated side of the neck.

Hair. It should be borne in mind that normal hair growth in the rat is characterized by alternate cycles of activity and inactivity. Furthermore, at any one time an inactive area may be adjacent to one of actively growing hair.

The hair follicles of the treated areas showed a picture of growth stasis (figs. 1 and 2, 3 and 4). In general, they extended down to the middle of the dermis and rarely did a follicle reach the panniculus adiposus. If it did, the follicle was small and inactive and in no case were growing hair bulbs observed. Epithelial buds were present but many of them seemed to be abnormal as compared with those in the areas of skin of the left side in which hair was not growing. In the former, the cells were smaller and their nuclei more compactly arranged (figs. 9 and 10). Sometimes the nuclei were of irregular shape and even pycnotic. Occasional evidence was obtained of actual atrophy of the inactive hair follicles as shown by loss of normal size and pycnosis of the

cells of the epithelial and connective tissue sheaths. In fact, it is probable that an actual reduction in number of follicles was elicited in some cases. This was most evident in biopsies removed after the longest period of treatment with 11-dehydro 17-hydroxycorticosterone (47 days) and the extract (55 days).

Epidermis. On the left (control) side, the epidermis was quite variable in its surface contour and thickness. Some of these irregularities probably resulted from contracture of the sample after excision. For the most part, three distinct regions could be delineated, namely, the stratum germinativum, granulosum and corneum. In all cases but one the treated area of the right side showed a definite thinning of the epidermis. The exceptional case had been treated for only 21 days but a slight reduction in thickness of the epidermis was indicated nonetheless. Such atrophic epidermis was characterized by a reduction in the number of cell layers in the stratum germinativum and diminution in size and flattening of the constituent cells (figs. 5, 6, 7, and 8). Sometimes, they were aggregated into clumps separated by stretches of extremely thin epidermis. The stratum granulosum, which on the left side was fairly prominent, was reduced greatly and frequently obliterated. In general, the stratum corneum was thinner after treatment but the extent of this change was difficult to evaluate.

Sebaceous glands. The sebaceous glands were exceedingly variable in number and size in the biopsies from the left side. With application of 11-dehydro 17-hydroxycorticosterone, no definite effect on the sebaceous glands could be ascertained as long as hair follicles were present. Towards the end of the experiment, if there was evidence of diminution in number of the hair follicles, then the sebaceous glands seemed to be reduced also.

Dermis. In general, the structure of the dermis in the treated areas was that of normal skin in which hair was not growing. The character of elastic fibers was unchanged. No clear evidence of interference with vascularity in histological preparations was apparent. The blood vessels located in the

panniculus adiposus were engorged with blood and capillaries were still present just beneath the epidermis. Mast cells appeared in considerable numbers especially in the deeper region of the dermis. Some evidence was secured of hyalinization of the collagenous fibers associated with degeneration of the connective tissue cells and pycnosis of cells in the sebaceous glands and in the sheaths of the hair follicles. The importance of this change cannot be evaluated since small patches of similar character occurred less frequently in control biopsies. However, such dermal modification seemed to increase with prolongation of the experiment, being most evident at 34 and 47 days. This suggests that if the treatment were continued beyond this period, degenerative changes in the dermis might be found to be a certain effect. The scarcity of the hormone prevented further study of this point.

The panniculus adiposus was reduced in thickness by 11-dehydro 17-hydroxycorticosterone as compared with areas of active hair growth in biopsies from the left side. Whether or not it was reduced to a significant degree beyond the state of normally inactive skin is problematical since some fat was generally present in treated areas.

The microscopic anatomy of the skin was not altered by application of 25% alcohol.

DISCUSSION

There is a considerable area of similarity in the effects on the skin of the parenteral injection of adrenocorticotropin (Baker, Ingle, Li and Evans, this journal) and percutaneous application of 11-dehydro 17-hydroxycorticosterone and an aqueous adrenal extract containing this and related cortical steroids. This resemblance was especially noticeable in the changes which occurred in the growth of the epidermis and hair. Likewise, there is no significant basic difference in the action of these substances as they were administered on the sebaceous glands. Thus, this information constitutes further evidence in support of the view that adrenocorticotropin prepared by the method of Li, Evans and Simpson ('43) stim-

ulates the release of at least some 11-oxysteroids by the adrenal cortex.

On the other hand, the increase in compactness of the dermis and the great reduction in the panniculus adiposus which followed the parenteral administration of adrenocorticotropin did not occur to as significant a degree after the percutaneous application of preparations containing adrenal steroids. One possible explanation for these differences may be that modification of the character of the dermis and the panniculus adiposus is dependent upon the general systemic action of the 11-oxysteroids. Thus, the injection of hypophyseal adrenocorticotropin stimulated the adrenals to produce them in quantities sufficient to exert a systemic effect while the effects of the hormone applied to the skin seemed to be limited to the area of application.

Much evidence is now available which shows that the adrenal cortex may modify growth processes to a significant degree. The relationship of adrenal cortical activity to the growth of hair was summarized previously. Ingle, Higgins and Kendall ('38) demonstrated that adrenal cortical extract (Cortin) inhibits the body growth of rats. Similar action was ascribed to corticosterone and 11-dehydro 17-hydroxycorticosterone, the latter compound being an unusually potent growth inhibitor (Wells and Kendall, '40b). Although adrenalectomy of young rats interferes with their growth (Hartman and Thorn, '30) when such rats are maintained on a salt regimen it has been possible to detect an increase in the width of the epiphyseal cartilage near the end of the second week after the operation (Wyman and Tum-Suden, '45). Wyman and Tum-Suden interpreted this effect as being due to accelerated secretion of the growth hormone by the hypophysis but, as they pointed out, it might also be explained on the basis of the release of the epiphyseal cartilage from the direct inhibitory action of adrenal steroids.

In addition, adrenalectomy has been reported to cause hypertrophy of lymphoid tissue (Reinhardt and Holmes, '40) although the data of Stoerek ('44) indicate that thymic hy-

perplasia does not follow adrenalectomy. Corticosterone and concentrated extract (cortin), but not desoxycorticosterone, cause thymic involution (Wells and Kendall, '40a). It should be noted from the work of Dougherty and White ('45) that the regulation of lymphocyte dissolution by the hormones of the adrenal cortex is probably an important factor in these modifications of lymphoid tissue. However, as yet one cannot exclude the control of hyperplasia as also being of importance. Some recently reported evidence tends to detract from the theory that lymphocyte dissolution is the sole cause of lymphoid atrophy in hyperadrenocortical states. Continued treatment with adrenal cortical steroids did not accelerate antibody production which indicates a failure of these substances to maintain continuous dissolution of lymphocytes (Eisen, Mayer, Moore, Tarr and Stoerck, '47). Although Nordenson ('47) reported the blood lymphocyte picture in man to be unaltered by treatment with adrenocorticotropin, a lymphopenia was observed by Forsham et al. ('48). These conflicting findings indicate that more attention should be given to the possibility that cortical steroids cause atrophy of lymphoid tissue by inhibiting the proliferation of cells as well as by causing their breakdown.

Biochemical information is available to provide some basis for explanation of the growth inhibition caused by the C-11 oxygenated steroids. Long, Katzin and Fry ('40) found that administration of these substances causes a rise in liver glycogen and in excretion of urinary nitrogen indicating increased protein catabolism and gluconeogenesis. The amount of nitrogen excreted under the influence of adrenal cortical steroids in their experiments was so great that these workers felt that some of the catabolized protein must have been derived from tissues other than the liver. As far as the epidermis and hair growth are concerned in our experiments not only does it appear that cellular proliferation had been inhibited by the 11-oxysteroids but, also, that an actual reduction in size of the epidermal cells had occurred. The observation that epithelial atrophy may be induced by the local application

of 11-oxysteroids also suggests a stimulation of protein catabolism in tissues other than the liver. Regardless of how protein loss may be involved in the inhibition of growth our results show that *protein catabolism in the liver is not prerequisite to inhibition of growth by 11-dehydro 17-hydroxycorticosterone or cortical extract.*

Until the present time it has not been clear whether adrenal cortical steroids inhibit growth directly or by inhibition of the secretion of growth hormone by the anterior hypophysis. Thus, after observing retarded body growth when corticosterone or 11-dehydro 17-hydroxycorticosterone was administered to growing rats, Wells and Kendall ('40b, p. 327) observed that "It has been suggested that certain effects of corticosterone are referable to depression of pituitary activity and it seems probable that the inhibition of growth by this and related compounds may have a similar explanation. . . . Whether this inhibition of growth is due simply to suppression of the production of the growth factor of the pituitary or to some direct influence of the compounds on metabolic processes is not clear." Our results do not rule out the possibility that injection or oral administration of cortical compounds may suppress release of the growth hormone. Nevertheless, they do demonstrate clearly that certain cortical steroids are *capable of suppressing growth of body tissue directly rather than by first inhibiting secretion on the part of the hypophysis or any other gland.*

Finally, the capacity of cortical steroids to inhibit growth by a peripheral action has a significant bearing upon another currently-held endocrine concept. White and Dougherty ('47) have postulated on the basis of data obtained in the mouse that adrenal steroids regulate the mobilization of protein from lymphoid tissue and that the thyroid gland performs the same function in respect to the carcass. Our experiments do not indicate the precise manner in which cortical steroids inhibit growth. Nevertheless, it seems reasonable to expect that ultimately the growth of cutaneous epithelial structures is stopped either by accelerating the loss of nitrogen from

them or by inhibiting their synthesis of it. If this be true, then the belief that the adrenal cortex mobilizes protein only from lymphoid tissue is open to question. As a corollary of their thesis, White and Dougherty postulated that cortical steroids may mobilize carcass nitrogen by first stimulating the thyroid. This, likewise, could not be used to explain the results which we obtained.

SUMMARY

The application of 11-dehydro 17-hydroxycorticosterone and aqueous adrenal extract to one side of the dorsum of the neck of rats was found to inhibit the growth of hair in the area treated. Microscopic examination of biopsy samples of the skin excised from treated and non-treated areas, showed that such treatment caused thinning of the epidermis and failure of hair follicles to develop beyond the stage of epithelial bud formation. These findings show that adrenal steroids may inhibit growth directly without the mediation of this action by any other endocrine gland or organ. Also, it is demonstrated that the catabolic action of the 11-oxysteroids on protein in the liver is not pre-requisite to their growth-inhibiting effects.

LITERATURE CITED

- BUTCHER, E. O. 1941 Accelerated hair growth in the rat after adrenalectomy cannot be attributed to the thyroid. *Proc. Soc. Exp. Biol. and Med.*, *48*: 120-122.
- BUTCHER, E. O., AND R. A. RICHARDS 1939 The relation of the adrenals to the retarded hair growth in underfed albino rats. *Endocr.*, *25*: 787-792.
- DOUGHERTY, T. F., AND A. WHITE 1945 Functional alterations in lymphoid tissue induced by adrenal cortical secretion. *Am. J. Anat.*, *77*: 81-116.
- EISEN, H. N., M. M. MAYER, D. H. MOORE, R. TARR AND H. C. STOERCK 1947 Failure of adrenal cortical activity to influence circulating antibodies and gamma globulin. *Proc. Soc. Exp. Biol. and Med.*, *65*: 301-306.
- FORSHAM, P. H., G. W. THORN, F. T. GARNET PRUNTY AND A. G. HILLS 1948 Clinical studies with pituitary adrenocorticotropin. *J. Clin. Endocr.*, *8*: 15-66.
- HARTMAN, F. A., AND G. W. THORN 1930 A biological method for the assay of cortin. *Proc. Soc. Exp. Biol. and Med.*, *28*: 94-95.

- INGLE, D. J., G. M. HIGGINS AND E. C. KENDALL 1938 Atrophy of the adrenal cortex in the rat produced by administration of large amounts of cortin. *Anat. Rec.*, **71**: 363-372.
- LI, C. H., H. M. EVANS AND M. E. SIMPSON 1943 Adrenocorticotrophic hormone. *J. Biol. Chem.*, **149**: 413-424.
- LONG, C. N. H., B. KATZIN AND E. G. FRY 1940 The adrenal cortex and carbohydrate metabolism. *Endocr.*, **26**: 309-344.
- NORDENSON, N. G. 1947 Clinical experimental observations on the influence of adrenocorticotrophic hormone (Corticotrophine) and desoxycorticosterone (DOCA) on the peripheral blood and the bone marrow. *Acta Med. Scand.*, **128**: (suppl. 196) 419-431.
- RALLI, E. P., AND I. GRAEF 1943 Stimulating effect of adrenalectomy on hair growth and melanin deposition in black rats fed diets adequate and deficient in the filtrate factors of vitamin B. *Endocr.*, **32**: 1-12.
- 1945 The effects of synthetic and natural hormone of the adrenal cortex on melanin deposition in adrenalectomized black rats fed diets adequate and deficient in the filtrate factors of vitamin B. *Endocr.*, **37**: 252-261.
- REINHARDT, W. O., AND R. O. HOLMES 1940 Thymus and lymph nodes following adrenalectomy and maintenance with NaCl in the rat. *Proc. Soc. Exp. Biol. and Med.*, **45**: 267-270.
- STOERCK, H. C. 1944 Thymus weight in relation to body weight in castrated and in adrenalectomized rats. *Endocr.*, **34**: 329-334.
- WELLS, B. B., AND E. C. KENDALL 1940a A qualitative difference in the effect of compounds separated from the adrenal cortex on distribution of electrolytes and on atrophy of the adrenal and thymus glands of rats. *Proc. Staff Meet., Mayo Clinic*, **15**: 133-139.
- 1940b The influence of corticosterone and C17 hydroxydehydrocorticosterone (Compound E) on somatic growth. *Proc. Staff Meet., Mayo Clinic*, **15**: 324-328.
- WHITAKER, W. L., AND B. L. BAKER 1948 Inhibition of hair growth by the percutaneous application of certain adrenal cortical preparations. *Science*, **108**: 207-209.
- WHITE, A., AND T. F. DOUGHERTY 1947 Role of the adrenal cortex and the thyroid in the mobilization of nitrogen from the tissues in fasting. *Endocr.*, **41**: 230-242.
- WYMAN, L. C., AND C. TUM-SUDEN 1945 The effect of adrenalectomy on the epiphyseal cartilage of the rat. *Endocr.*, **36**: 340-346.

PLATE 1

EXPLANATION OF FIGURES

All preparations illustrated on this page were strip biopsies stained with Harris' hematoxylin and eosin. $\times 60$.

1 Left (non-treated) side of neck of W63. Hair is in a state of active growth and the bulbs are surrounded by a well-developed panniculus adiposus. Bouin fixation.

2 Right side of neck of W63 treated for 21 days with 11-dehydro 17-hydroxy-corticosterone. Hair growth is stopped, the panniculus adiposus is reduced although a significant amount is still present. Sebaceous glands are still large. Bouin fixation.

3 Left side of neck of BH45. 3a is of an area of active hair growth with a thick panniculus adiposus and 3b of an inactive area with a reduced but still significant panniculus adiposus. Carnoy fixation.

4 Right side of neck of BH45 treated with aqueous adrenal extract for 48 days. Hair is not growing and the panniculus adiposus is reduced. The lightly stained area below the hair follicles consists of hyalinized connective tissue. Carnoy fixation.

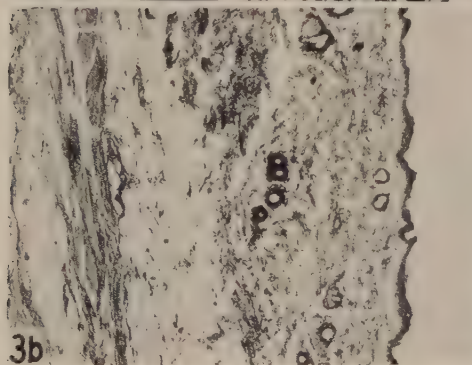
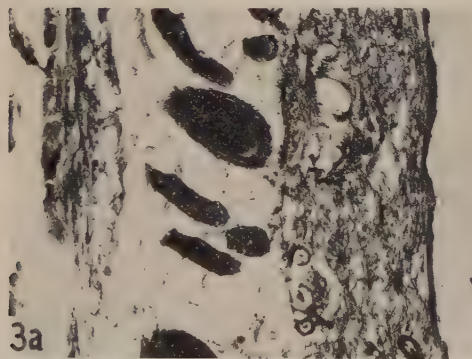
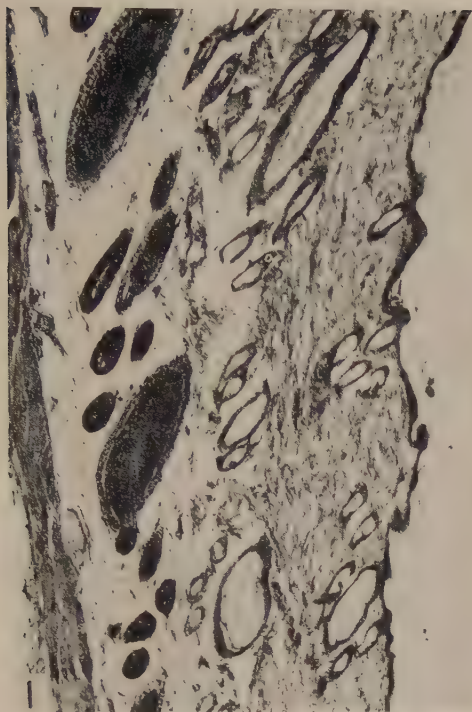


PLATE 2

EXPLANATION OF FIGURES

5 Punch biopsy, left side of neck of BH64. The epidermis is thick, the stratum germinativum consists of several layers of cells and the stratum granulosum is present. 10 μ . Bouin fixation. Hematoxylin and eosin. $\times 272$.

6 Punch biopsy, right side of neck of BH64, treated with 11-dehydro 17-hydroxycorticosterone for 24 days. The epidermis is thinner, the cells are smaller and the stratum granulosum greatly reduced. Technique and magnification as in figure 5.

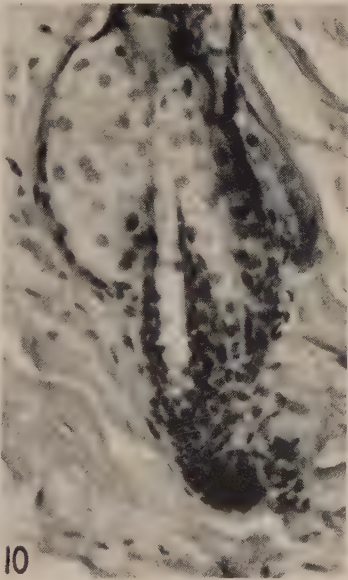
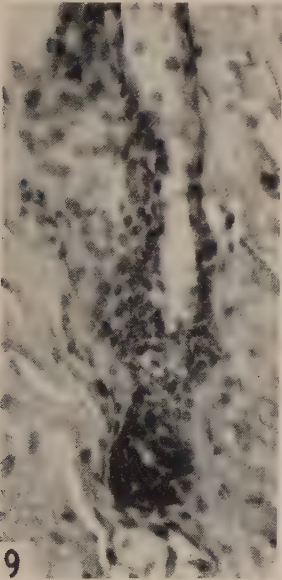
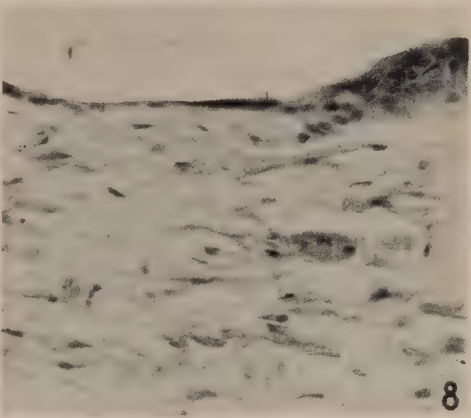
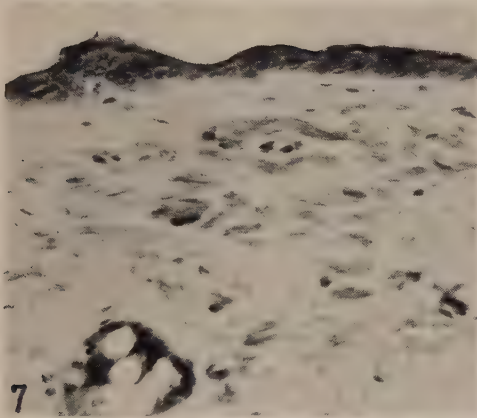
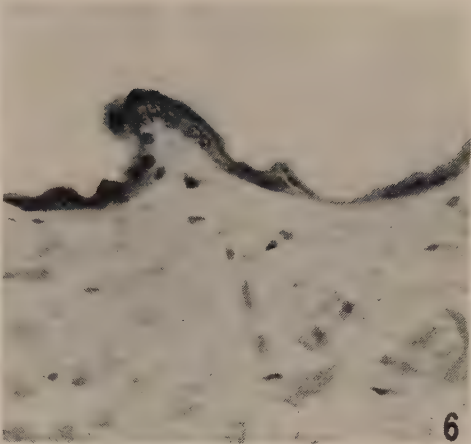
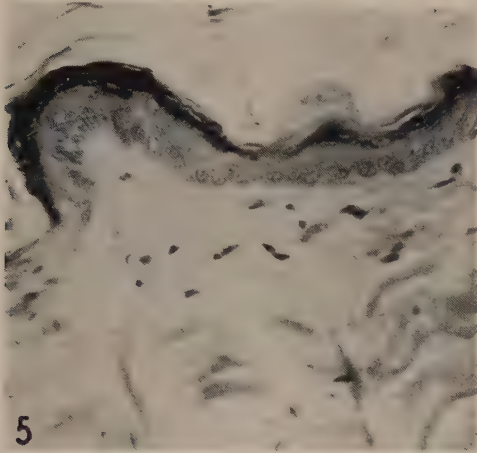
7 Strip biopsy, left side of neck of BH54. The epidermis is several cell layers thick and the stratum granulosum is present. 10 μ . Carnoy fixation. Hematoxylin and eosin. $\times 272$.

8 Strip biopsy, right side of neck of BH45 treated with adrenal extract for 48 days. The epidermis is greatly thinned. At the right is a clumping of epidermal cells, these aggregations being observed frequently in treated areas. Technique and magnification as in figure 7.

9 Left side of neck of W63. Epithelial bud at the lower end of a hair follicle. 10 μ . Hematoxylin and eosin. $\times 296$.

10 Right side of neck of W63 treated for 21 days with 11-dehydro 17-hydroxycorticosterone. The epithelial bud at the lower end of the hair follicle illustrates the stage in which hair growth was stopped. The cells composing it are dense and atrophic as compared with those illustrated in figure 9. Technique and magnification as in figure 9.

11 The area of skin caudal to the right ear of this rat had been treated with adrenal extract for 48 days. Notice the asymmetry in the hair growth pattern created by the inhibition in the treated area.



GLYCOGEN AND CARBOHYDRATE-PROTEIN COMPLEXES IN THE OVARY OF THE WHITE RAT DURING THE OESTROUS CYCLE

B. T. HARTER

*Departments of Pathology, University of Illinois College of Medicine, (Chicago)
and of Anatomy, The Johns Hopkins Medical School, Baltimore*

SIX FIGURES 

An investigation of the carbohydrate moieties of the ovary of the white rat during the oestrous cycle was undertaken because it was felt that certain modifications of the techniques of previous workers would yield information hitherto unobtainable. While this work was in progress, Wislocki, Bunting, and Dempsey ('47), published their observations on metachromasia and the Bauer reaction in the ovaries of the sow and rat. Both of these methods aid in visualizing certain fractions of the total family of substances which contain reactive carbohydrate moieties, but these are not yet clearly definable (Wislocki, Bunting, and Dempsey, '47; Hotchkiss, '48). It was felt also that the effort of the former investigators to make these methods more specific, by the use of hyaluronidase prior to staining, was limited by the mode of fixation they employed. It was thought worthwhile, then, to continue our systematic study in which somewhat different techniques were being used.

We employed the following major modifications of the procedure used by earlier workers:

1. All ovaries were fixed by freezing and drying in order to reduce the artefacts due to alterations in protein structure.

2. The periodic acid-leucofuchsin method of McManus ('46) and of Hotchkiss ('48) was employed to visualize all polysaccharides and protein or lipid-bound reactive carbohydrate (i.e., glycogen, glycoprotein, and glycolipid, resp.). The method was selected because it will demonstrate, with few exceptions, all carbohydrates containing free alcohol groups in the two and three positions of the carbohydrate ring or chain. These carbohydrates are stained a pink to deep bluish-red color, depending upon the number of reactive groups present. The total carbohydrate thus visualized included those substances which are made apparent by metachromatic staining and by the Bauer reaction, as well as others (Lillie, '48).
3. The total reactive material was then separated into classes of compounds by the use of extractives, enzymes, or other reagents, which were applied to sections of tissue in which the proteins were relatively undenatured during the process of fixation by freezing and drying. It was possible in this way to distinguish clearly between the glycoprotein present in different sites.

Because of these modifications we were able to touch upon a number of additional problems such as: the origin of the glycoprotein components of the follicular fluid and zona pellucida, the dispersal of the cells of the corona radiata during fertilization, the penetration of the zona pellucida by the spermatozoon, and the appearance and disappearance of glycogen in the ovum.

MATERIALS AND METHODS

From a selected group of white rats that showed regular oestrous cycles over a 30-day period, three or 4 were killed by thoracotomy on each day of the 5-day cycle. The ovaries were immediately removed and immersed in iso-pentane maintained at -150°C . After freezing they were transferred to a vacuum chamber at about -30°C ., dehydrated, and embedded in paraffin (m.p. 56° – 58°C .). This procedure is believed to be superior to methods of preparation commonly employed

because it avoids undue denaturation of proteins, minimizes post-mortem enzymatic deterioration, and reduces movement of more mobilizable components.

The ovaries were sectioned at 6μ and the sections were applied to the slide by pressure. They were then warmed until the paraffin was just melted and again flattened by pressure. Paraffin was removed by immersing the slide in petroleum ether for 5 minutes. To determine the total reactive polysaccharide and glycoprotein, the deparaffinized section was immersed in absolute alcohol for 20 hours to fix the tissue, then stained by the periodic acid-leucofuchsin method (which stains the carbohydrate complexes red), counterstained with malachite green, and mounted in clarite. In order to rule out glycolipids, control sections were incubated in a mixture of equal parts methanol and chloroform at 37°C . for 20 hours to extract lipids. As no change in the intensity or distribution of the stain could be detected after this extraction, we concluded that no appreciable amount of glycolipid was present in the sections and that the stained substances were free carbohydrates or those bound to protein. To determine solubility, the deparaffinized section was covered by 5 drops of the reagent for a definite period of time (varying with the reagent) at room temperature, washed with 5 drops of buffer solution (having the same pH as the reagent), and immersed in absolute alcohol for 20 hours. Subsequent staining as described above for the total reactive substances served to identify which component had been extracted by the reagents employed. The following agents were employed for extraction purposes:

1. Buffer solutions were applied to the section for 60 minutes. The pH range was 2.0, 4.5, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0. The buffers were prepared with KCl-HCl, acetate, phosphate, and borate.
2. Hyaluronidase was applied for 30 minutes. It was prepared from bull testis extract by dissolving 1 mg of the purified enzyme in 1 ml of buffer at pH 4.5 and at pH 8.0.

3. Pepsin was applied for 30 minutes. The powdered enzyme (Pepsin, Difco Laboratories, 1:10,000) was dissolved in buffer at pH 4.5; 1 mg per 1 ml.
4. Trypsin was applied for 4 minutes. It was prepared from pancreatin dissolved in a buffer at pH 8.0 and activated by enterokinase. The final solution contained 1 mg of trypsin per 1 ml.
5. Saliva was applied to the sections for 60 minutes. Human saliva was filtered and diluted 1:10 with buffers at pH 4.5 and pH 8.0. Material not extracted by the buffers alone, but extracted by the buffered saliva, was considered to be glycogen.

RESULTS

The growth of the ovarian follicle has been divided arbitrarily into three stages: Stage I. The ovum is surrounded only by one to three layers of follicular cells and there is no zona pellucida and no antrum. Stage II. A zona pellucida is present, but no antrum has formed. The follicle is considerably larger than in Stage I and the intercellular cement may be heavier in some places, but otherwise the appearance is the same. Stage III. The follicle is still larger and a follicular antrum is present which contains follicular fluid.

Distribution of total polysaccharide and glycoprotein in the ovary during oestrus

Germinal epithelium. Fine pink granules are present in the cytoplasm. The cells are separated from one another by brighter walls of intercellular cement substance, and from the ovary by a dark red, broad basement membrane.

Follicles. Stage I (primary follicles). The granular or finely reticulated cytoplasm of the ovum is stained deep red, while the nucleus is completely devoid of color. The cytoplasm of the follicular cells contains a few, fine, lightly staining granules. The intercellular substance is visible as faint pink lines. The basement membrane is homogeneous and deep red.

Stage II (zona pellucida present, no antrum; fig. 1). The fibrillar cytoplasm of the ovum is deep red. The cytoplasm appears to be somewhat less deeply colored in this stage than in earlier or later stages. It is surrounded by an equally red zona pellucida which appears as a fibrillar network even coarser-meshed than that of the ovum. In earlier stages of development, the cytoplasm of the follicular cells contains a few small, pale pink granules. In later stages, just preceding antrum formation, some of the cells, two or three layers removed from the ovum, contain several larger, more deeply staining granules. The intercellular substance around most of the follicular cells appears as faint pink lines, but around those cells which contain the larger granules it is markedly thicker and more deeply staining. The basement membrane appears to be similar to that of younger follicles.

Stage III (containing an antrum; figs. 2 and 6). Except for the increased size of the ovum, the stainable material appears similar to that in the previous stage. The most prominent change is related to the antrum formation and to the cumulus oöphorus. As the antrum appears, the adjoining granulosa cells are notable for their increased number of large, deep red cytoplasmic granules. Such cells become more numerous as the antrum becomes larger. Other follicular cells, nearer the basement membrane or the ovum, still contain only a few, fine, pink staining granules. Similarly, when the cumulus appears, the cells closer to the antrum are densely packed with coarse, deep red granules (fig. 4), while those bordering on the ovum contain only a few delicate granules. In the last period of follicular growth, just preceding the time of ovulation, all the cells of the cumulus acquire numerous large granules (fig. 5). At this final period of maturation the follicular cells away from the cumulus contain only a few, if any, large granules. The precipitable components of the follicular fluid appear as a uniform, deep pink, shredded matrix whose fibrillar structure is a result of ice crystal formation during freezing, and of sectioning (fig. 5). The intercellular material appears in sections as delicate pink lines

between the follicular cells; it is a deeper pink, and somewhat thicker between cells which contain the coarser granules and are closer to the antrum. The basement membrane is homogeneous and appears to stain less intensely and to become notably thinner, sometimes discontinuous, in the period just preceding ovulation.

Atretic follicles (fig. 6). The cytoplasm of the ovum is refractile, granular or finely reticulated, and much more deeply stained than in normal ova. Macrophages which may invade the follicle and ovum are loaded with large granules of similarly staining material. The zona pellucida, when present, has a similarly enhanced color, and its reticulated structure is less coarse than in normal follicles. The granulosa cells contain large refractile granules. The basement membrane is thickened and appears to be fibrillar.

Corpus luteum. The cytoplasm of the luteal cells contains fine, pale pink granules. The intercellular substance appears as somewhat deeper pink, delicate lines. Macrophages containing yellow pigment granules are frequently found to contain numerous dark-red granules.

Blood vessels. The blood vessels are prominently stained. The red color is restricted to the internal elastic membrane and to the cement substance between the muscle cells of the media.

As far as we were able to differentiate, there was no essential deviation from this general description at any time during the oestrous cycle; however, in a single section there might be some variation in intensity on either side of that which has been described. All of the intercellular substance in the ovary was stained, but we concerned ourselves only with those sites described above.

*Characteristics of the carbohydrate-containing
stainable components of the ovary*
(See tables 1, 2, 3)

Ovum. Some of the reactive material in the cytoplasm of the normal egg cell is dissolved in buffers at pH 2.0 and 4.5;

TABLE 1

Relative solubilities of glycoproteins in cells and extracellular substances of the rat ovary in buffer solutions. Increasing amounts of total reactive material remaining after extraction are indicated by + to + + + +, with relation to a standard which consists of preparations in which minimal or no extraction had taken place. When a trace of reactive material is present, it is indicated by the ± signs. When no reactive glycoprotein was present following extraction, it is indicated by the — sign.

	TOTAL REACTIVE MATERIAL	pH 2.0	pH 4.5	pH 6.0	pH 7.0	pH 8.0	pH 9.0	pH 10.0	pH 11.0
STROMA OF OVARY									
Stage I	++	+	+	—	—	—	—	—	—
Stage II	++	±	+	—	—	—	—	—	—
Stage III	++	±	+	—	—	—	—	—	—
Atretic	+++ (+)	±	±	++	++	++	++	++	++
Zona pellucida	++	—	—	+	+	+	+	+	+
FOLLICLE									
Cumulus and antral cells (large granules)	+++ (+)	+	+	+	+	+	+	+	+
Other follicular cells (small granules)	+	—	±	±	±	—	—	—	—
Intercellular cement	+	±	±	—	—	—	—	—	—
Follicular fluid	++	+	+	—	—	—	—	—	—
Basement membrane	+	+	+	+	+	+	+	+	+
Basement membrane of atretic follicles	++	+	+	+	+	+	+	+	+
Luteal cells	+	±	±	±	±	±	±	±	±
Intercellular cement	++	+	+	+	+	+	+	+	+
Macrophages	+++	±	+	+	+	+	+	+	+
Macrophages of atretic follicles	+++	+	+	+	+	+	+	+	+

TABLE 2

Relative extractabilities of glycogen and glycoproteins in cells and extracellular substances of the rat ovary by use of saliva, hyaluronidase, pepsin and trypsin. The signs have the same significance as in table 1. The reference point is the glycoprotein present in the structures treated with buffer alone under the same conditions.

	pH 4.5				pH 8.0			
	BUFFER	SALIVA	HYALURONIDASE	PEPSIN	BUFFER	SALIVA	HYALURONIDASE	TRYPSIN
CYTOPLASM OF								
Stage I	+	±	+	++	—			
Stage II	+	±	+	+	—			
Stage III	+	±	+	+	—			
Atretic	±	±	±	—	++	++	++	++
Zona pellucida	—				++	++	++	++
Cumulus and antral cells								
(large granules)	+	+	+	+				
Other follicular cells								
(small granules)	±	±	—	—				
Intercellular cement	±	±	—	—				
Follicular fluid	+	+	+	+				
Basement membrane	+	+	+	±	+	+	±	+
Basement membrane of								
atretic follicle	+	+	+	+	+	+	+	++
Luteal cells	±	±	±	±	±	±	—	—
Intercellular cement	+	+	±	+	+	+	±	±
Macrophages	+	+	+	+	+	+	±	+
Macrophages of								
atretic follicles	+	+	+	+	+	+	+	++

all of it is dissolved in more alkaline solutions. In the atretic ova more of the reactive material is extracted at lower pH values (2.0 and 4.5), but in buffers at pH 6.0 and above very little is removed. Saliva appears to remove some of the material from normal ova, though it has no apparent effect on atretic ova. This is the reverse of the action of pepsin, which appears to remove some of the stainable material from the atretic ova, though none is removed from normal ova. Trypsin has no apparent effect on the concentration of stainable material in the atretic ova. Thus, the cytoplasm of the normal ovum contains a rather uniformly distributed, readily soluble glycoprotein and glycogen. The cytoplasm of the atretic ovum contains a glycoprotein with different properties, since it is quite soluble at low pH values, but is rather insoluble at pH 6.0 and above; it differs also in that it appears to have no detectable glycogen. Hyaluronidase has no effect on these glycoproteins.

Zona pellucida. The reactive material in the pellicle of normal and atretic ova is completely removed by buffers of pH 2.0 and 4.5, is partially removed at pH 6.0–9.0, and is not affected at pH 10.0 and 11.0. It is not affected by trypsin, hyaluronidase, or saliva.

Follicles. In general, the glycoproteins of the following structures behave similarly to buffers: the faint pink, minute granules of the granulosa cells, the intercellular substance between the granulosa cells, and the reactive material of the follicular fluid. In all cases, much or all of the stainable material is soluble throughout the pH range employed, although less so in the more acid solutions. The glycoprotein of large, deep red granules in the cells of the cumulus and the cells bordering the antrum is somewhat less soluble but shows the same decrease in solubility in more acid solutions. It is difficult to assess the effects of hyaluronidase. This enzyme appears to cause a loss of glycoprotein in follicular cells containing small granules, and a decrease in those cells which contain large granules. It also causes a loss of intercellular glycoprotein without affecting the solubility of the

reactive material of the follicular fluid. The isolated appearance of the granulosa cells resulting from removal of the intercellular glycoprotein is very striking. Pepsin results in similar but not identical extractions. Saliva causes a decrease in the intensity of the large, deeply staining granules in the cytoplasm of the cells of the cumulus and the cells bordering the antrum. This suggests that glycogen may be present in these cells in very small amounts. The basement membrane of the normal follicles is somewhat affected by all buffers in the range employed, but is more markedly affected at pH 9.0 or higher. On the other hand, in the atretic follicles, the glycoprotein of the basement membrane appears to be very slightly soluble at pH 6.0 and 7.0 and somewhat more soluble at pH's lower and higher, but not to the same extent that the basement membrane of the normal follicles is soluble in the latter instance of the higher pH's. Pepsin digests the basement membrane of normal follicles and hyaluronidase appears to remove some of it, but trypsin does not appear to affect it. None of these enzymes seems to have any effect on the basement membrane of the atretic follicle.

Corpus luteum. The faintly stainable granules are rather soluble in all buffers, and are completely soluble at pH 9.0 and above. Trypsin and hyaluronidase also appear to remove some reactive material. The intercellular substance behaves like the basement membrane of normal follicles, in that it is soluble in all buffers and is more soluble in the range pH 9.0–11.0. It is similar also in that hyaluronidase appears to assist in the removal of some of the glycoprotein. On the other hand, the action of pepsin and trypsin is just reversed.

The glycoprotein component of the inclusions of the macrophages in the corpus luteum is very largely extracted by buffers at pH 2.0 and 9.0–11.0 and is partially extracted at intermediate values. Hyaluronidase and pepsin cause the solution of some of this material, while trypsin has no apparent effect. These observations differ markedly from those made on the glycoprotein component of the inclusions in the macrophages in the atretic follicles. Here the material is

less readily affected by both buffers and enzymes, although hyaluronidase has a questionably positive effect.

DISCUSSION

The results show clearly that glycogen is present in the cytoplasm of the normal ovum. Its distribution appears to be uniform, in contradistinction to the peculiar localization described by Wislocki and his colleagues ('47). Also distributed uniformly in the cytoplasm is a rather soluble glycoprotein. A carbohydrate is present in small amounts in the germinal epithelium as discrete granules. With the methods employed we were not able to characterize this small amount of reactive material in the germinal epithelium. During the development of the follicle, the total number of reactive groups recognizable by the method employed is high in the ovum before the zona pellucida is formed (Stage I). It is also high after the antrum has developed (Stage III). In between is a period when the number of reactive groups is somewhat lower (Stage II).

One of the classical procedures of cytology in the separation and characterization of cell structures is the method of differential solubility by the use of fixatives, some of which preserve one or another structure specifically while not preserving others. In the work reported here, the differential solution of a family of chemical substances (carbohydrate-containing proteins) was attempted, and to a certain degree, achieved, in various structures of the rat ovary. The results are summarized briefly in table 3. The remaining part of this discussion will be concerned with some of the implications of these observations.

One of the surprising things is that in atretic ova, which are devoid of detectable glycogen, the glycoprotein appears to have peculiar properties. It is either a different glycoprotein, which is more difficultly soluble than that of normal ova, or it is the same or similar glycoprotein in a different state of polymerization. An even more remarkable feature is the observation that the glycoprotein of atretic ova bears a striking resemblance in its solubilities and extractability to that

of the zona pellucida. This simple characterization of the two glycoproteins is scarcely sufficient to establish their relationship; but it may serve to indicate that this component of the zona pellucida may be a product of the ovum, which normally is extruded from the ovum, but is either overproduced or retained in part in the process of atresia. An addi-

TABLE 3

Differential solubilities of glycoproteins of the cells and extracellular substances of the rat ovary

GLYCOPROTEIN	BUFFER pH 2.0-4.5	BUFFER pH 6.0-8.0	BUFFER pH 9.0-11.0	HYALUR- ONIDASE	SALIVA	PEPSIN
Cytoplasm of ovum	Present	Absent	Absent	Present	Largely absent	Present
Zone pellucida	Absent	Present	Present	Present	Present	
Cytoplasm of atretic ovum	Largely absent	Present	Present	Present	Present	Absent
Small granules of follicular cells and intercellular cement of follicle	Largely absent	Largely absent	Absent	Absent	Present	Absent
Large granules of follicular cells around antrum and in cumulus oöphorus	Present	Largely absent	Largely absent	Present	Present	Present
Follicular fluid	Present	Absent	Absent	Present	Present	Present
Basement membrane and intercellular cement of corpus luteum	Present	Present	Largely absent	Largely absent	Present	Present
Cytoplasm of cells in corpus luteum	Largely absent	Largely absent	Absent			

tional link between the ovum and the zona pellucida is the observation that at the time of appearance of stainable glycoprotein in the pellicle there is very little detectable glycoprotein in the adjacent follicular cells.

It is interesting to speculate on the mode of penetration of the relatively inert and resistant zona pellucida by the

fragile spermatozoa. The glycoprotein component of the zona pellucida appears to be more soluble in rather acid media (pH 4.5) than in other reagents employed. It is well known that motile spermatozoa liberate carbon dioxide and lactic acid (Comstock, '39; Henle and Zittle, '42). This suggests that the spermatozoon may penetrate the pellicle by creating around itself a more acid medium which dissolves the pellicle locally, allowing it to advance through the barrier.

It will be recalled that the cytoplasm of the ovum was described as less fibrillar in appearance in younger ova (Stage I) than in older ova (Stage II and III). Atretic ova were also characterized by a more delicate cytoplasmic network. On the other hand, the zona pellucida is always more highly reticulated. It is possible that submicroscopic intrinsic structure of the protein components may influence the degree of reticulation. It is, however, more likely that the fibrillar pattern represents largely the result of ice crystal formation during the freezing of the organ in iso-pentane. During the freezing of homogeneous suspensions, ice crystals are larger in more dilute preparations, and smaller in more concentrated ones, other factors being equal. These observations suggest the possibility that the water concentration is lower in younger ova and in atretic ova than in older ova, and that the concentration of water in the zona pellucida is higher than in the ovum at any stage of development.

The follicular cells of the graafian follicle appear to have some glycoprotein granules at all stages of follicular maturation. At all stages, also, the cement substance between them contains glycoprotein. When the antrum is formed it is filled with follicular fluid which contains glycoprotein. That these substances are related to one another is suggested at the time of antrum formation, and at the time of most rapid growth in the size of the follicle during oestrus. Just before the antrum is formed and in the early stages of its formation, the cells in the immediate vicinity have large, deeply stained granules. Preceding ovulation, the granulosa cells in the cumulus oöphorus and in the corona radiata also have simi-

larly prominent glycoprotein granules. These increases in the number of cells with large granules at the times of follicular fluid production indicate that they may be secretion antecedents, which are poured into the follicular cavity. That the large and small granules of the follicular cells may be different is indicated by several observations. The solubility and extractability of the two glycoproteins appear to be different. The glycoprotein of the small granules has extractability properties which resemble closely those of the glycoprotein of the intercellular cement. The most striking observation which indicates two different glycoproteins in the follicular cells is the action of hyaluronidase on the glycoproteins of the intercellular cement and of the follicular fluid. Short exposure to hyaluronidase readily removes the cementing substance that binds the granulosa cells, especially noticeable in the cumulus. This is in agreement with the findings of McClean and Rowlands ('42), Fekete and Duran-Reynals ('43), and Leonard and Kurzrok ('45). These authors reported that hyaluronidase causes the coronal cells to be dispersed more readily. It was expected that the glycoprotein component of the follicular fluid would have similar properties to those of the cement substance, but this was not the case. The surprising finding is that the glycoprotein component of the former is not affected by hyaluronidase. It is difficult to reconcile this refractoriness to hyaluronidase with the observation of Wislocki and his group ('47) that metachromasia of the follicular fluid disappears after treatment with the enzyme. Possible explanations of the difference are: (1) the time of action of the enzyme was too short in the experiments reported here, (2) the glycoprotein of the cement substance is altered as it leaves its cellular environment, (3) the follicular cells are capable of elaborating two different glycoproteins under different stimuli (the antecedents being the large and small granules observed).

The basement membrane of the graafian follicle is of some interest. Throughout the growth of the follicle, from its first appearance to the later stages preceding ovulation, it seems

to retain the same homogeneous staining properties and thickness. This indicates that the basement membrane is in fact constantly growing, for in no other way would it be possible for it to retain the same thickness. Just preceding ovulation, it becomes thinner and appears discontinuous in some regions. This final thinning of the basement membrane is further evidence of its plastic nature, and may represent a stage in the sequence of events preceding ovulation.

SUMMARY AND CONCLUSIONS

Paraffin sections of the rat ovary in different stages of the oestrous cycle were prepared by freezing and drying. The sections were then treated by the periodic acid-leucofuchsin reagents to demonstrate glycogen and carbohydrate-containing proteins. An effort was made to characterize some of the glycoproteins by means of extractives and enzyme preparations. Glycoprotein was visible in the germinal epithelium, ovum, zona pellucida, follicular granulosa cells, follicular fluid, cells and macrophages of the corpus luteum, intercellular substance, basement membrane, and atretic ovum. It was possible to show that glycoproteins in the various sites can be extracted differentially, and are presumably distinct from each other. By this method it is clear that glycoprotein in the normally developing ovum (which is the only cell containing appreciable amounts of glycogen) has different properties from that in the atretic ovum. There appears to be a clear relationship between increased cytoplasmic glycoprotein of the granulosa cells and antrum formation and growth. The reactions of the zona pellucida to extractives suggest that penetration by the spermatozoon may be effected by local changes in hydrogen ion concentration. The ready removal of glycoprotein of the follicular cells and of the cement substance between them by hyaluronidase suggests this glycoprotein as the substrate which is acted upon by the enzyme when it causes accelerated dispersal of the cells of the corona radiata.

ACKNOWLEDGMENTS

I am indebted to Dr. Granville A. Bennett for extending to me the courtesies of his laboratories, and to Dr. Isidore Gersh for his counsel. The work was supported in large part by a grant of the American Cancer Society recommended by the Committee on Growth, National Research Council.

REFERENCES CITED

- COMSTOCK, R. E. 1939 A study of the mammalian sperm cell. I. Variations in the glycolytic power of spermatozoa and their relation to motility and its duration. *J. Exp. Zool.*, *81*: 147.
- FEKETE, E., AND R. DURAN-REYNALS 1943 Hyaluronidase in the fertilization of mammalian ova. *Proc. Soc. Exp. Biol. Med.*, *52*: 119-121.
- HENLE, G., AND C. A. ZITTLE 1942 Studies of the metabolism of bovine epididymal spermatozoa. *Am. J. Physiol.*, *136*: 70.
- HOTCHKISS, R. D. 1948 A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. *Arch. Biochem.*, *16*: 131.
- LEONARD, S. L., AND R. KURZROK 1945 A study of hyaluronidase — effects on the follicle cells of ovulated rat ova. *Endocrinology*, *37*: 171.
- LILLIE, R. D. 1948 Histochemical observations incident to a study of the mucins. Symposium on the Applications of Chemical Methods to Pathology, 37th meeting of the International Association of Medical Museums, American and Canadian Section.
- MCCLEAN, D., AND I. W. ROWLANDS 1942 Role of hyaluronidase in fertilization. *Nature*, *150*: 627-628.
- MCMANUS, J. F. A. 1946 The histological demonstration of mucin after periodic acid. *Nature*, *158*: 202.
- WISLOCKI, G. B., H. BUNTING AND E. W. DEMPSEY 1947 Metachromasia in mammalian tissues and its relationship to mucopolysaccharides. *Am. J. Anat.*, *81*: 1.

PLATE

PLATE 1

EXPLANATION OF FIGURES

1 Early Stage II. Graafian follicle in the ovary of a white rat. The zona pellucida is just forming at the periphery of the ovum. The nucleus of the ovum is unstained. Intercellular cement and basement membrane can be seen as thin gray lines. Stained with periodic acid-leucofuchsin. Magnification $\times 700$.

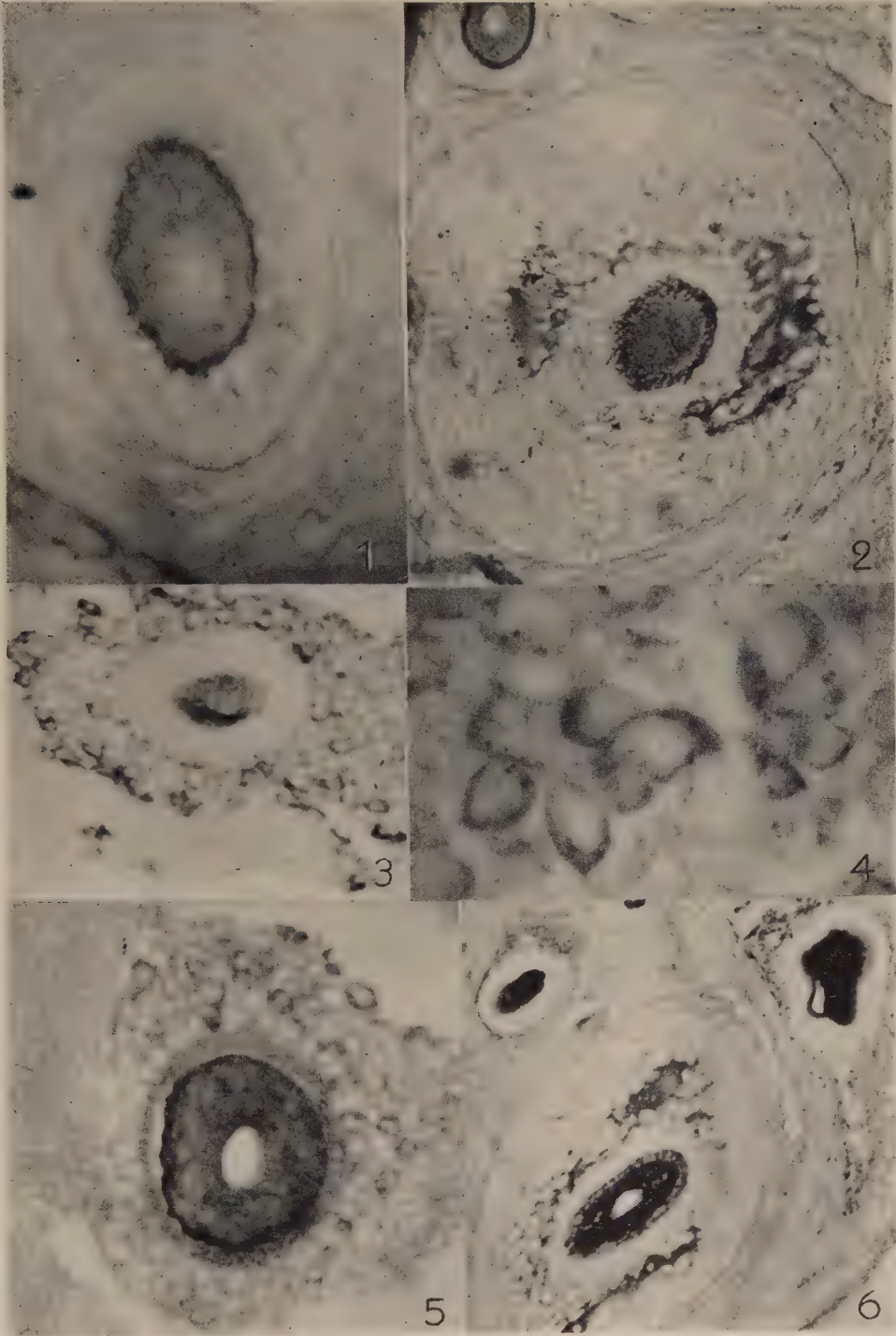
2 Stage III. Graafian follicle in ovary of white rat. The section through the ovum which is in the center of the follicle is tangential, so that no nucleus appears; a darker zona pellucida encloses it. The follicular fluid is visible as areas of gray patches between the follicular cells. The intercellular cement and basement membrane are visible as in figure 1. Magnification $\times 190$.

3 A typical example of a cumulus oöphorus extracted before staining by buffer at pH 4.5. The outstanding change is the loss of stainability of the zona pellucida. Other changes are indicated in table 1. Magnification $\times 380$.

4 Granulosa cells in the cumulus oöphorus of a follicle. Large, deeply stained granules are present in the cytoplasm of the cells. Magnification $\times 1280$.

5 Cumulus oöphorus of a follicle that is in the last period of maturation. The nucleus of the ovum is unstained, the cytoplasm is reticulated, and the zona pellucida is reticulated and fibrillar. The cytoplasm of all the granulosa cells, including those adjacent to the ovum, is loaded with deeply staining granules. The follicular fluid is seen as a fibrillar reticulum to the left of the ovum in the figure. Magnification $\times 700$.

6 Two atretic follicles are seen in the upper part of the figure. The atretic ovum on the right still has a lighter staining zona pellucida present. In the center of the figure is a follicle which is in Stage III. Magnification $\times 190$.



IMPLANTATION OF TUMORS IN THE HIND LIMB FIELD OF THE EMBRYONIC CHICK AND THE DEVELOPMENTAL RESPONSE OF THE LUMBOSACRAL NERVOUS SYSTEM¹

ELMER D. BUEKER

*Department of Anatomy, Schools of Medicine and Dentistry,
Georgetown University, Washington, D. C.*

SIX FIGURES

It has been experimentally demonstrated in embryos that the periphery (mesodermal and ectodermal derivatives as represented in developing limbs and the body wall) is essential for the differentiation of sensory and motor centers of the central nervous system and the sensory ganglia. This was done by increasing the periphery (Detwiler, '20, '30, '36; Schwind, '31; Hamburger, '39; Bueker, 45a; Piatt, '46) as in transplantation experiments or by decreasing the periphery as in extirpations and then observing the developmental response in the nervous system (Braus, '06; Shorey, '09; Durken, '11; Detwiler, '20, '36; May, '30, '33; Stultz, '42; Hamburger, '34; Hamburger and Keefe, '44; Bueker, '43; Hall and Schneiderhan, '45; Barron, '43, '45; Piatt, '46). Results of workers in this field are not all in agreement but general results indicate that the non-neural periphery is essential. The nature of the stimuli residing in the periphery which exert this influence are largely unknown. However, a brief survey of some of the evidence which indicates that this interrelationship exists appears to be both profitable and necessary.

During normal development there is a predilection of somatic motor nerve fibers for developing muscle, and of

¹ Aided by a grant from the Penrose Fund of the American Philosophical Society.

sensory nerve fibers for developing sensory structures. In the outgrowth of spinal nerves (or any mixed nerve), sensory and motor fibers are in common connective sheath; and yet their distal terminations are always in correct relationship with the periphery. Taylor ('44) has demonstrated that the removal of spinal ganglia in late larval stages of amphibia will result in pure muscle innervation; removal of the spinal cord will result in pure sensory innervation. To account for this phenomenon one may postulate as does Taylor ('44) the presence of local physicochemical differentiation in peripheral structures which cause proper nerve fibers to make correct terminal connections.

The influence of a denervated area on peripheral nerves is well known. A periphery without nerves attracts outgrowing nerve fibers. This attraction continues until it is again innervated. Once innervated, nerve fibers are no longer admitted and it cannot be overloaded (Fort, '40). Stimuli for attracting nerves have been shown to exist to such degree in denervated amphibian limbs that peripheral nerves have been demonstrated to grow through circuitous routes to innervate such limbs (Hamburger, '29).

Although experimental evidence on the nature of the interaction between the periphery and outgrowing nerves with their cells of origin is fragmentary, the explanation given by Weiss ('41) is most acceptable. The direction of pioneer nerve fibers of embryonic neurons or regenerating nerve stumps is first determined by the structural arrangement of the substratum. A few of the fibers by chance make the right terminal connections. Presumably physicochemical affinities residing in the periphery are responsible for making these connections. After connections are made the surface properties of fibers change so as to attract adjacent fibers to form a nerve bundle. This is known as selective fasciculation. Physicochemical changes in the fibers must in some way be projected to the cell bodies for, without this, degeneration will occur. With connections intact, cells of origin continue to differentiate until mature.

A hypothetical explanation of the interaction of the non-neural periphery with the nervous system during development may therefore be summarized as follows: (1) stimuli reside in the periphery; (2) physicochemical changes occur in nerve fibers as a result of their relationship with this periphery; (3) changes in the fibers are projected to the cells of origin and are thus responsible for cellular differentiation. After the periphery has imposed its influence on a neuron and its fibers, it seems that surrounding neurons may be influenced to differentiate because of inductive changes which occur while pioneer fibers are attracted during selective fasciculation, or from influences emanating from primary differentiated cell bodies (Hamburger, '34; Hamburger and Keefe, '44) or their dendrites (Barron, '43, '45).

Peripheral stimuli necessary for the differentiation of sensory and motor centers of the spinal cord and spinal ganglia are in some of their characteristics highly non-selective. The hind limb periphery in chick embryos is adequate for the development of brachial or thoracic motor neurons (Bueker, '45a). Cranial nerves will innervate muscles of transplanted limbs in amphibians (Detwiler, '30). Finally, the statement is made that any motor nerve may innervate any muscle (Weiss, '41). This does not exclude the possibility of gradations of preference of certain nerve fibers for given muscles.

It is also true, using birds as experimental animals, that the limb periphery of another genus may serve as an adequate stimulus for the differentiation of spinal ganglia and motor cells (Bueker, '45a). Grafting of spinal cord segments between different species of amphibia with successful peripheral innervation has been accomplished (Wiemann, '26; Detwiler '31, '32) or limb rudiments may be grafted heteroplastically (Schwind, '31). The fact that sensory and motor cells need not be matched with their normal architecture and that the periphery of another species or genus is adequate for their differentiation is evidence of a high degree of non-specificity. In foreign peripheral environments, motor and sensory fibers

are still found to terminate in their proper muscle and sensory surroundings.

Stimuli which are essential for the differentiation of anterior horn cells and spinal ganglion cells exert their influence in chick embryos, as shown experimentally, from the 5th to the 9th days of total incubation (Hamburger, '44, Bueker, '45b), and must reside in a highly complex rapidly differentiating cellular environment. Connective tissue is still in primitive mesenchymal form but probably biochemically determined and muscle elements are in myoblast stages. At present the complexity of this histogenetic environment is such as to defy analysis.

It is proposed that experiments be performed in which this complex periphery be partly or entirely removed at a given level of the nervous system, as in limb ablation experiments in chick embryos, and cells of given genetic origin and with fixed properties be substituted. This was attempted in the investigation presented. The hind limb was entirely or partly removed and various tumors substituted. Tumor cells were selected for the following reasons: (1) they maintain their autonomy; (2) grow rapidly; (3) have more or less fixed histogenetic properties which make it possible to classify them as sarcomas, carcinomas, etc. A given tumor should present a uniform histogenetic picture if it has been carefully selected for this purpose, and changes in the nervous system of embryos may then be linked with peripheral stimuli which reside in a given cellular environment.

I wish to express my deep appreciation to Dr. Morris Belkin of the National Cancer Institute for supplying the tumors used in this investigation.

METHOD

Chick embryos of 26–34 somites were used as hosts in these experiments (usually of 50–60 hours incubation). The

hind limb primordium is located in the somatopleure lateral to somites 24-32, extending as far as the future amniotic fold. In earlier experiments the entire limb field was extirpated, while in later experiments a strip of somatopleure lateral to somites 24-32, having approximately the width of somites at this level, was removed. Tumors are then implanted in the removed area. It was found that the distal portion of the limb somatopleure was needed to anchor the implanted tumor.

Mouse mammary adenocarcinoma, mouse sarcoma 180, and Rous fowl sarcoma were used as transplants (for growth studies of mammalian tumors in chick embryos see Rous and Murphy, '11; Hungate and Snider, '45). Tumors were removed and placed in 0.9% sterile saline. This material was then cut into small pieces with a razor blade. The site of implantation was examined and a piece of tumor tissue selected which fitted snugly into this area. After transplantation the opening in the egg was covered with a glass cover slip and the edges sealed with paraffin. General methods for transplanting in chick embryos were outlined by Hamburger, '42.

Operated embryos were observed at 24-hour intervals during the first three days postoperatively to determine the fate of transplants. By making these observations it is possible to select material which will be most useful for intensive study. Six specimens were selected for each of the three series of tumor transplants and fixed in Bouin's fluid as follows: one at 4, one at 6, and 4 at 8-9 days' total incubation. The lower half of each specimen was imbedded, sectioned at 10μ and stained with Heidenhain's hematoxylin.

Cases presented in table 1 were studied by making graphic reconstructions of lumbosacral peripheral nerves on the normal and experimental sides to determine the degree of peripheral innervation and to identify spinal cord segments (fig. 1). Motor cells were counted on both sides of the lumbosacral spinal cord (every other section counted) and cardboard reconstructions made from camera lucida drawings of lumbosacral spinal ganglia. Weighings of reconstructions

were recorded in grams (table 1). Cell counts were made of ganglia 22-30 of case A-12 on right and left sides counting every other section. Similar counts were made of spinal ganglia 27-30 of A-11, and 23-26 of Rs-14. Only cells with nuclei were counted.

The lumbosacral peripheries of A-1 (4 days' total incubation and A-12 (8 days' total incubation) were projected. Drawings were made of the malformed limb with the imbedded tumor and the contralateral normal limb. Outline drawings were cut out and weighed to determine volumes of the tumor and limb substance on the experimental side and compared with the volume of the normal limb.

RESULTS

Fifty transplants were made of each of the following: Mouse mammary adenocarcinoma, mouse sarcoma 180, and Rous fowl sarcoma (total, 150 experiments). The mortality was 52, 62, and 86%, respectively. Except for Rous fowl sarcoma, death during the first two days postoperatively was usually caused by bacterial contamination. Embryos with Rous fowl sarcoma transplants developed extensive hemorrhagic areas and only a few survived 8-9 days' total incubation. This high mortality was due to Rous fowl sarcoma virus which causes hemorrhage in chick embryos (Milford and Duran-Reynals, '43).

Mouse mammary adenocarcinoma transplants (Ca-series) were gradually absorbed and tumor cells were not found in sectioned material of 8-9 days' total incubation. In the two cases studied, Ca-1 and Ca-4 (table 1), the limbs on the experimental side were malformed with a total volume of approximately $\frac{1}{2}$ to $\frac{2}{3}$ of the contralateral normals. Graphic reconstructions of peripheral nerves showed nerves of the lumbosacral plexus greatly reduced in size, and irregularities in the plexus were present, which can be associated with limb malformations. Lateral motor columns were reduced 59.1 and 62.8%, respectively, in the above mentioned cases. Spinal ganglia were reduced 36% in volume in each of the two

TABLE 1

Spinal ganglia in grams (cardboard reconstructions) and counts of lateral motor neurons of the lumbosacral segments on the experimental side (E) as compared with the normal side (N)

TYPE OF TRANSPLANT		SPINAL CORD SEGMENTS										TOTAL	DIFF. %
		22	23	24	25	26	27	28	29	30			
Sarcoma 180													
A-11 (8-d)	G	E	4.62	5.87	9.16	8.14	7.37	6.40	6.23	6.55	5.02	59.36	+ 23.2
		N	4.18	5.59	6.82	5.28	5.72	5.95	6.06	4.95	3.65	48.20	
	M	E	530	1006	994	1360	716	993	650	685	441	7375	— 23.9
		N	544	1052	1002	1730	1388	1339	1119	849	663	9686	
A-12 (8-d)	G	E	10.36	11.30	11.15	10.40	9.51	8.70	7.31	6.27	5.66	80.66	+ 40.4
		N	7.15	5.96	6.30	8.05	7.66	6.74	6.41	4.71	4.46	57.44	
	M	E	643	685	630	399	478	421	458	470	211	4395	— 43.8
		N	720	923	1069	1218	997	1052	898	545	403	7825	
A-20 (9-d)	G	E	7.69	8.72	11.58	12.96	10.28	9.75	9.73	7.18	7.05	84.94	+ 35.6
		N	7.08	7.67	5.75	8.13	10.05	7.25	6.18	5.58	4.95	62.54	
	M	E	520	852	968	583	508	363	360	172	373	4699	— 38.0
		N	569	1024	1201	1320	1120	1056	544	351	400	7585	
Av.	G												+ 33.1
	M												— 35.2
Rous sarcoma													
Rs-12 (8-d)	G	E	5.20	3.14	3.32	2.76	3.25	4.66	4.63	3.93	3.85	34.74	— 41.4
		N	7.55	6.78	5.45	9.55	6.80	7.64	5.73	5.63	4.17	59.30	
	M	E	63	160	268	473	647	932	311	252	201	3307	— 49.0
		N	170	413	1136	1233	1192	1241	552	301	251	6489	
Rs-14 (8-d)	G	E	4.95	5.63	6.05	8.85	7.40	4.25	4.92	3.92	3.25	49.22	— 24.9
		N	4.75	6.82	8.65	10.35	7.85	10.07	6.55	4.95	5.53	65.52	
	M	E	430	424	309	233	349	446	236	144	156	2727	— 55.5
		N	933	875	688	993	1216	747	344	153	175	6124	
Rs-15 (9-d)	G	E	6.52	6.00	5.73	6.10	6.55	6.80	5.85	5.05	4.75	53.35	— 35.0
		N	10.10	10.00	8.70	11.55	12.66	10.30	7.35	6.03	5.75	82.44	
	M	E	301	141	158	175	255	311	347	197	170	2055	— 68.6
		N	603	1011	992	1200	843	940	488	245	230	6552	
Av.	G												— 33.8
	M												— 55.7
Tumor absorbed													
Ca-1 (9-d)	G	E	4.55	4.98	5.25	6.15	4.90	3.85	4.45	4.25	3.90	42.28	— 36.2
		N	7.39	7.04	6.88	11.80	9.05	7.80	7.25	5.25	3.80	66.27	
	M	E	195	328	343	417	375	344	208	212	172	2594	— 59.1
		N	332	733	872	1327	1032	1004	563	300	171	6334	
Ca-4 (9-d)	G	E	6.03	6.64	7.12	6.03	6.51	5.94	4.93	4.62	5.39	53.21	— 36.0
		N	10.34	7.04	13.40	14.74	10.00	9.68	5.72	5.50	6.71	83.13	
	M	E	184	266	279	340	337	280	245	160	111	2202	— 62.8
		N	644	1087	1325	1075	769	425	301	199	101	5926	
A-21 (9-d)	G	E	7.45	6.33	6.28	5.57	4.25	4.68	4.13	2.65	5.33	46.67	— 34.7
		N	7.88	7.71	8.06	5.05	8.25	11.67	12.45	4.45	5.95	71.47	
	M	E	256	485	674	735	734	630	288	683	346	4831	— 37.4
		N	285	500	752	822	1185	1321	1116	1058	677	7716	
Av.	G												— 35.6
	M												— 53.2

(E, experimental side; N, normal side; G, weight of cardboard reconstructions of spinal ganglia in grams; M, number of neurons in the lateral motor column, every other section counted; +, hypertrophy and hyperplasia; —, hypoplasia.)

cases (table 1). Reductions in size of peripheral nerves, of spinal ganglia, and in number of cells of the lateral motor columns were usually in proportion to the extent of limb removal and the degree of malformation and agree generally with results obtained in limb ablation experiments (Hamburger, '34).

Mouse sarcoma 180 (A-series) grows exceedingly well in chick embryos. One case, A-21, of 6 specimens sectioned was without tumor cells. Observations on embryos during the course of the experiment indicated that transplants, when not too large, generally grow and blend into their surroundings, are covered with skin of the host, and are not seen in fixed specimens of 8-9 days' total incubation. Three cases, A-11, A-12, and A-20 (table 1), retained well developed transplants. In each case the tumor was incorporated in the limb field and accounted for rather striking effects on the developing nervous system.

Case A-11. Tumor tissue was imbedded in the malformed limb and replaced that portion normally occupied by the hamstring, gluteal and adductor muscles. The distal portion of this limb was present as an aborted stump. Some of the tumor cells had infiltrated medially into the mesonephros and laterally into the stump. Contralaterally the limb was normal. Spinal nerves from ganglia 22 and 23 of the malformed limb were not extending into the tumor but into limb substance and the volume of the ganglia and number of cells in the lateral motor column were approximately symmetrical (table 1). Most of the peripheral innervation from segment 24 projected into limb substance; two small branches could be traced from this segment caudally into the tumor. Motor cells in the lateral column were approximately equal on the two sides of the spinal cord of this segment, while the spinal ganglion was hypertrophied. Spinal nerves from segments 25-30 had grown into the tumor. In these segments there was a hypoplasia of the lateral motor column and a hypertrophy of spinal ganglia. The average increase in vol-

ume for the spinal ganglia (segments 22-30), was 23.2%, while the lateral motor column was reduced 23.9% (table 1).

Case A-12. The distal portion of the hind limb was missing on the experimental side. Tumor tissue incorporated in this structure occupied 47.5% of its total volume (table 2). Its distribution lateral to segments 22-30 showed that 72-88% of the periphery was tumor in the lower levels (table 2, fig. 5). Volume of the tumor and malformed limb combined was 78% of the contralateral normal. All of the lumbosacral spinal

TABLE 2

Abnormal limb, sarcoma 180, and normal limb of A-12 (in grams from paper reconstructions) lateral to segments 22-30

SEGMENTS	N	E		D
		L	S	%
22	4.403	3.230	0.080	2.4
23	3.221	2.015	0.205	9.2
24	2.935	1.535	0.870	35.0
25	4.600	2.368	1.285	35.2
26	4.085	1.183	1.160	49.5
27	3.420	0.556	1.986	78.2
28	2.486	0.263	1.186	81.9
29	2.430	0.280	2.080	88.1
30	1.870	0.760	1.990	72.3
Totals	29.450	12.190	10.842	47.5

(N, normal limb; E, abnormal limb with sarcoma 180; L, limb substance in abnormal limb; S, sarcoma 180; D, per cent sarcoma of L and S combined.)

nerves on the experimental side were traced into the tumor (figs. 1, 6). All the spinal ganglia were enlarged as compared with contralateral normals. According to the distribution of tumor tissue one would expect a higher percentage of enlargement of ganglia in the lower segments. However, such a correlation was not found (tables 1 and 2). Nerves which coursed through the tumor were large proximally, become very much branched and ended as fine arborizations between the tumor cells (figs. 1,6).

The lateral motor column was hypoplastic in all segments, average 43.8% (fig. 5) while the ganglia were enlarged, average 40.4% (table 1).

Case A-20. Tumor tissue was incorporated in a malformed limb and occupied most of the space normally filled by the hamstring and adductor muscles. General location of the tumor was similar to A-11. The contralateral limb was normal. Tumor cells had infiltrated into the mesonephros and distal portion of a malformed stump. Peripheral nerves from segments 22 and 23 projected into limb tissue; the spinal ganglia and lateral motor columns were almost symmetrical in these segments (table 1). Horizontal branches from segments 24 and 25 projected into limb substance while caudally directed branches extended into the tumor. Large nerves from segments 26-30 extended into the tumor. In all segments with tumor innervations the spinal ganglia were hypertrophied and the lateral motor column hypoplastic; the averages were 35.6 and 38.0%, respectively (table 1).

Case A-21. No trace of the tumor transplant was found. The hind limb on the experimental side was malformed and the total limb material was of the same order as the Ca-series reported in table 1. The contralateral limb was normal. Peripheral nerves were reduced in all segments except those from 22 and 23. Reduction in size of spinal ganglia and of cells in the lateral motor column can be explained on the basis of results of partial limb removal, average reduction in volume of the ganglia 34.7% and of the lateral motor column 37.4%.

Spinal ganglion cells were counted in every other section on the experimental and normal sides of A-12 from ganglion 22-30 and a total of 42,168 was obtained on the experimental side as compared with 39,532 contralaterally (figs. 1, 5). The differences in per cent between the two sides in individual segments (22-30) was: -0.72, +23.7, +33.9, +5.9, +3.1, -3.8, +0.5, +11.6 and -5.9, respectively; average +6.4 (+ means an increase in cell number on the experimental side). Except for segments 23 and 24 with increases of 23.7 and

33.9% over the normal side, the differences are probably within the error for counting. The cellular increase of 23.7 and 33.9% was associated with volume increase of 98.6 and 77.0%. In A-11, spinal ganglia 27-30 were counted, the total on the experimental side was 18,268 as compared with 17,137 contralaterally. The differences in per cent (27-30) were: +3.5, -5.9, +3.8, +17.3; average +6.6. All differences were within experimental error in counting except 30 with an increase of 17.3 in favor of the experimental side. Except for the three ganglia mentioned above, the evidence for a hyperplasia is not convincing and the increase in size of the ganglia on the experimental sides of A-11, A-12, and A-20 must be accounted for largely on the basis of an increase in size of the cellular elements or a hypertrophy (table 1, fig. 4).

Rous fowl sarcoma experiments Rs-12, Rs-14, and Rs-15 each had malformed limbs on the experimental side. In this malformed structure considerable tumor tissue was present. Massive hemorrhagic areas were found in all the sectioned material. The tumor is not pure histologically, as in A-11, A-12 and A-20, but it is composed of muscle and diverse connective tissue elements. Considerable infiltration of the tumor occurred beyond the limb region through the mesonephros of the experimental side and extending often to the opposite side of the mid-line. Peripheral nerves from all lumbosacral segments were considerably reduced and the lumbosacral plexus irregular. With a reduction in peripheral nerves was associated a reduction in volume of the spinal ganglia and cells of the lateral motor column of 33.8 and 55.7% (an average for the three cases, table 1). The presence of the tumor had no influence on the development of the nervous system and the general results agree with those obtained in A-21, Ca-1, and Ca-4 in which tumors failed to develop.

Spinal ganglion cells were counted in 23-26 of Rs-14 on the experimental and normal sides; the total was 18,201 and 23,111 respectively, or a cellular decrease of 21.1% on the experimental side. This may be interpreted as a hypoplasia. The decrease in volume of spinal ganglia 22-30 of Rs-14 of the

experimental side as compared with the normal side was 24.9%. Volume reduction in this case is therefore a reduction of cell number, a hypoplasia. From general observation on spinal ganglia of A-21, Ca-1, Ca-4, Rs-12, and Rs-15 in the lumbosacral level, one may well conclude that the reduction in size of the ganglia is accounted for by a hypoplasia. This is typical of results obtained in limb ablation experiments on chick embryos.

Generally, in cases of 4 to 5 days' total incubation, except where tumors were absorbed or dislocated, the volume of the tumor and the malformed limb combined was greater than the normal limb periphery. In one case, A-1 (fig. 2), of 4 days' total incubation, the weights of paper models in grams of the experimental and normal peripheries were 1.16 and 0.78 respectively. During the 6 to 9 days' (figs. 3, 5) total incubation, the growth rate of the normal limb is such that its volume generally exceeds the experimental periphery (table 2). This observation is important inasmuch as it must be considered later in the interpretation of results.

DISCUSSION

1. *Control experiments (Ca-1, Ca-4, A-21).* In these experiments tumors failed to grow. The amount of limb material removed, the amount of tumor tissue implanted and the mechanical disturbances associated with transplanting were similar to the other cases presented in table 1 in which tumors had developed. They were therefore considered as controls. In each case of this series the limb was malformed with a volume reduction within the range of one-third to one-half of the normal. Peripheral nerves supplying this structure were likewise smaller and highly irregular. Spinal ganglia and lateral motor columns were hypoplastic. These results were in general agreement with those obtained by partial limb ablation (Hamburger, '34).

2. *Rous fowl sarcoma experiments (Rs-12, Rs-14, Rs-15)* Conditions were similar to control experiments except for the presence of well-developed Rous fowl sarcoma. General

reduction in volume of the malformed limb and the tumor combined was in each of the three cases within the range of malformed limbs of controls. All nerves were reduced in size and there was no difference between those innervating limb substance and those innervating the tumor. Spinal ganglia and lateral motor columns were hypoplastic on the experimental side (table 1).

The presence of Rous fowl sarcoma had no apparent influence on the developing lumbosacral nervous system and the general picture is one of reduction of nervous elements as in controls. Three factors are suggested to explain why the presence of this tumor had no apparent effect: (1) the tumor was not homogeneous histologically and consisted of a mixture of muscle and connective tissue elements; (2) tumor cells did not remain localized in the limb field as well as sarcoma 180 and there was considerable infiltration to surrounding structures; (3) massive hemorrhagic areas were present which may have interfered with growth processes.

3. *Mouse sarcoma 180 experiments (A-11, A-12, A-20)*. General factors associated with transplanting and limb ablation were similar to controls. The tumors (sarcoma 180) did not disappear as in controls and were well developed in three cases (A-11, A-12, A-20; table 1, fig. 5). Volumes of limb substance and tumor combined were in each case within the range of four-fifths to two-thirds of the contralateral normal limb (table 2). As was mentioned previously the experimental periphery with the tumor is generally larger than the normal periphery during the first two or three days post-operatively (fig. 2), and then becomes less during the 6-9 day total incubation period (figs. 3, 5).

Sarcoma 180 transplants remained localized to the limb field and were well innervated (figs. 1, 5, 6). This tumor was uniformly composed of cells which appear histologically undifferentiated and has a history of many generations of transplantation in mice. It differs from Rous fowl sarcoma in having less divergent histogenetic elements, and there was no vascular disturbance such as was observed in the Rs-series.

It is believed that sarcoma 180, because of intrinsic physicochemical properties and mechanics of growth, selectively causes the enlargement of spinal ganglia. This is suggested by the following facts (1) all ganglia with peripheral nerves extending into the tumor were larger than contralateral normals; (2) this enlargement was consistent and cannot be explained on the basis of chance variations (table 1); (3) in control experiments, except for the absence of the tumor, factors were similar to the A-series and the ganglia were in all cases hypoplastic; (4) in the Rs-series tumors were present and peripheral nerves extended from ganglia into the tumors but all ganglia were hypoplastic.

It is particularly interesting that the lateral motor column was hypoplastic in the same segments in which the ganglia were enlarged and this is consistent in the three cases presented (table 1). One may assume that this difference would not have occurred under conditions where the periphery is equally favorable for the outgrowth and development of sensory and motor elements for in such cases there is a hypoplasia of spinal ganglia and the lateral motor column when the periphery is decreased as in limb ablation experiments (Hamburger, '34), or a hyperplasia of both as in limb transplantation experiments (Hamburger, '39). The fact that spinal ganglia were enlarged while the lateral motor column was reduced in those segments which innervated sarcoma 180 suggests quite definitely that this tumor selectively favored the development of one and not the other.

Cross sections of material at 4 days' total incubation show that the experimental periphery with sarcoma 180 was larger than the contralateral normal (fig. 2). This observation was supplemented by others made on living embryos during the course of the experiments and the general indications were that the limb field with its tumor was greater in volume during the 3-5 day total incubation period. However, sectioned material showed no enlargement of the ganglia during this time. There is some indication that the motor column had undergone a slight hypoplasia.

During the 6-9 day total incubation period the experimental periphery becomes less in volume than the normal side. In A-12 reconstructions of the periphery show that the tumor and malformed limb combined represent 78% of the volume of the normal limb (table 2). This experiment was terminated at 8 days' total incubation. This proportionate reduction in volume for A-11 and A-20 on the experimental side was of a similar order. Increase in size of the ganglia occurred largely during the 6-9 day interval and it is highly significant that this increase occurred in the presence of a periphery which was actually less than the normal in volume. The indications are that enlargement of ganglia with a decreased periphery would not have been possible without a selective periphery such as was afforded by this tumor. In conclusion one may state that sarcoma 180 presented a periphery with mechanical and histochemical properties which favored sensory innervation and hence the enlargement of spinal ganglia. The enlargement was largely one of increase in cell size (hypertrophy) with some increase in cell number (hyperplasia).

SUMMARY

A portion or the entire hind limb field was removed unilaterally from 50-60 hour chick embryos. Mouse adenocarcinoma, mouse sarcoma 180, and Rous fowl sarcoma were implanted in embryos where limb material had been removed. The influence of tumors on the development of the lumbosacral nervous system was studied at 4-9 days' total incubation. The results obtained are given below.

1. Mouse mammary adenocarcinoma transplants were absorbed. Limbs were malformed on the experimental side and reduced one-third to one-half the volume of the contralateral limb. Peripheral nerves, spinal ganglia and the lateral motor column were reduced in accordance with results obtained in limb ablation experiments.

2. Rous fowl sarcoma transplants were well developed. Various histogenetic elements were found in this tumor. No effect was observed on the development of the nervous system

and results were in general agreement with those obtained in mouse mammary adenocarcinoma experiments.

3. In mouse sarcoma 180 experiments the spinal ganglia which innervated this tumor were enlarged 33% (average of three cases) above those on the control side, while the lateral motor columns were hypoplastic.

4. It is postulated that sarcoma 180 transplants were responsible for the hypertrophy and hyperplasia observed in the lumbosacral ganglia because of intrinsic physicochemical properties and mechanics of growth.

LITERATURE CITED

- BARRON, DONALD H. 1943 The early development of the motor cells and columns in the spinal cord of the sheep. *J. Comp. Neur.*, 78: 1-27.
- 1945 The role of sensory fibers in the differentiation of the spinal cord in sheep. *J. Exp. Zool.*, 100: 431-443.
- BRAUS, H. 1906 Vordere Extremität und Operculum bei Bombinator-Larven. *Morph. Jahrb.*, 35: 509-590.
- BUEKER, ELMER D. 1943 Intracental and peripheral factors in the differentiation of motor neurons in transplanted lumbosacral spinal cords of chick embryos. *J. Exp. Zool.*, 93: 99-129.
- 1945a The influence of a growing limb on the differentiation of somatic motor neurons in transplanted avian spinal cord segments. *J. Comp. Neur.*, 82: 335-361.
- 1945b I. Unilateral limb extirpation and its effect on the lumbosacral spinal cord. Year Book of the American Philosophical Society, 1945, pp. 173-175.
- DETWILER, S. R. 1920 On the hyperplasia of nerve centers resulting from excessive peripheral loading. *Proc. Nat. Acad. Sci.*, 6: 96-101.
- 1930 Observations upon the growth, function, and nerve supply of limbs when grafted to the head of salamander embryos. *J. Exp. Zool.*, 55: 319-328.
- 1931 Heteroplastic transplantations of embryonic spinal cord segments in *Amblystoma*. *J. Exp. Zool.*, 60: 141.
- 1932 Growth acceleration and regulation in heteroplastic spinal cord grafts. *J. Exp. Zool.*, 61: 245.
- 1936 *Neuroembryology, An Experimental Study*, New York, The Macmillan Company.
- DÜRKEN, B. 1911 Über frühzeitige Extirpation von Extremitätenanlagen beim Frosch. *Zeitschr. wiss. Zool.*, 99: 189-355.
- FORT, W. B. 1940 An experimental study of the factors involved in the establishment of neuromuscular connections. University of Chicago Libraries, Chicago, Illinois.

- HALL, E. K., AND M. A. SCHNEIDERHAN 1945 Spinal ganglion hypoplasia after limb amputation in the fetal rat. *J. Comp. Neur.*, 82: 19-34.
- HAMBURGER, V. 1929 Experimentelle Beiträge zur Entwicklungsphysiologie der Nervenbahnen in der Froschextremität. *Archiv. f. Enwickmech.*, Bd. 119, S. 47.
- 1934 The effect of wing bud extirpation on the development of the central nervous system in chick embryos. *J. Exp. Zool.*, 68: 449-494.
- 1939 Motor and sensory hyperplasia following limb-bud transplantations in chick embryos. *Physiol. Zool.*, 12: 268-284.
- 1942 *A Manual of Experimental Embryology*. The University of Chicago Press, Chicago, Ill.
- HAMBURGER, VIKTOR, AND EUGENE L. KEEFE 1944 The effects of peripheral factors on the proliferation and differentiation in the spinal cord of chick embryos. *J. Exp. Zool.*, 96: 223-242.
- HUNGATE, R. E., AND HESTER SNIDER 1945 Experiments of factors inhibiting the growth of mammalian tumors in eggs. The University of Texas Publication, 109-117.
- MAY, R. M. 1930 Reprécussions de la greffe de moelle sur le système nerveux chez l'embryon de l'Anoure, *Discoglossus pictus*. *Bull. Biol. France et Belg.*, 64: 355-387.
- 1933 Réactions neurogéniques de la moelle à la greffe en surnombre ou à l'ablation d'une ébauche de patte postérieure chez l'embryon de l'Anoure, *Discoglossus pictus*. *Ibid.*, 67: 327-349.
- MILFORD, JOHN J., AND F. DURAN-REYNALS 1943 Growth of a chicken sarcoma virus in the chick embryo in absence of neoplasia. *Cancer Research*, 3: 578-584.
- PIATT, JEAN 1946 The influence of the peripheral field on the development of the mesencephalic V nucleus in *Amblystoma*. *J. Exp. Zool.*, 102: 109-142.
- ROUS, PEYTON, AND J. B. MURPHY 1911 Tumor implantation in the developing embryo. *J.A.M.A.*, 56: 741.
- SHOREY, M. C. 1909 The effect of the destruction of peripheral areas on the differentiation of the neuroblasts. *J. Exp. Zool.*, 7: 25-63.
- SCHWIND, J. L. 1931 Heteroplastic experiments on the limb and shoulder girdle of *Amblystoma*. *J. Exp. Zool.*, 59: 265-295.
- STULTZ, WALTER A. 1942 Alterations in the spinal cord of *Amblystoma* following changes in the peripheral field. *Anat. Rec.*, 82: 450 (abstr.).
- TAYLOR, A. CECIL 1944 Selectivity of nerve fibers from dorsal and ventral roots in the development of the frog limb. *J. Exp. Zool.*, 96: 159-185.
- WIEMAN, H. L. 1926 The effect of heteroplastic grafts of the spinal cord on the development of the limb in *Amblystoma*. *J. Exp. Zool.*, 45: 335-348.
- WEISS, PAUL 1941 Nerve patterns: the mechanics of nerve growth. *Growth, Third Growth Symposium*, 5: 163-203.

PLATE 1

EXPLANATION OF FIGURE

1 Graphic reconstruction of the lumbosacral peripheral nerves of A-12 (8 days' total incubation) Branching on the left caused by mouse sarcoma 180. L.M.C., cell counts of the lateral motor column (every other section). Sp.G., cell counts of the spinal ganglia (every other section).

A-12

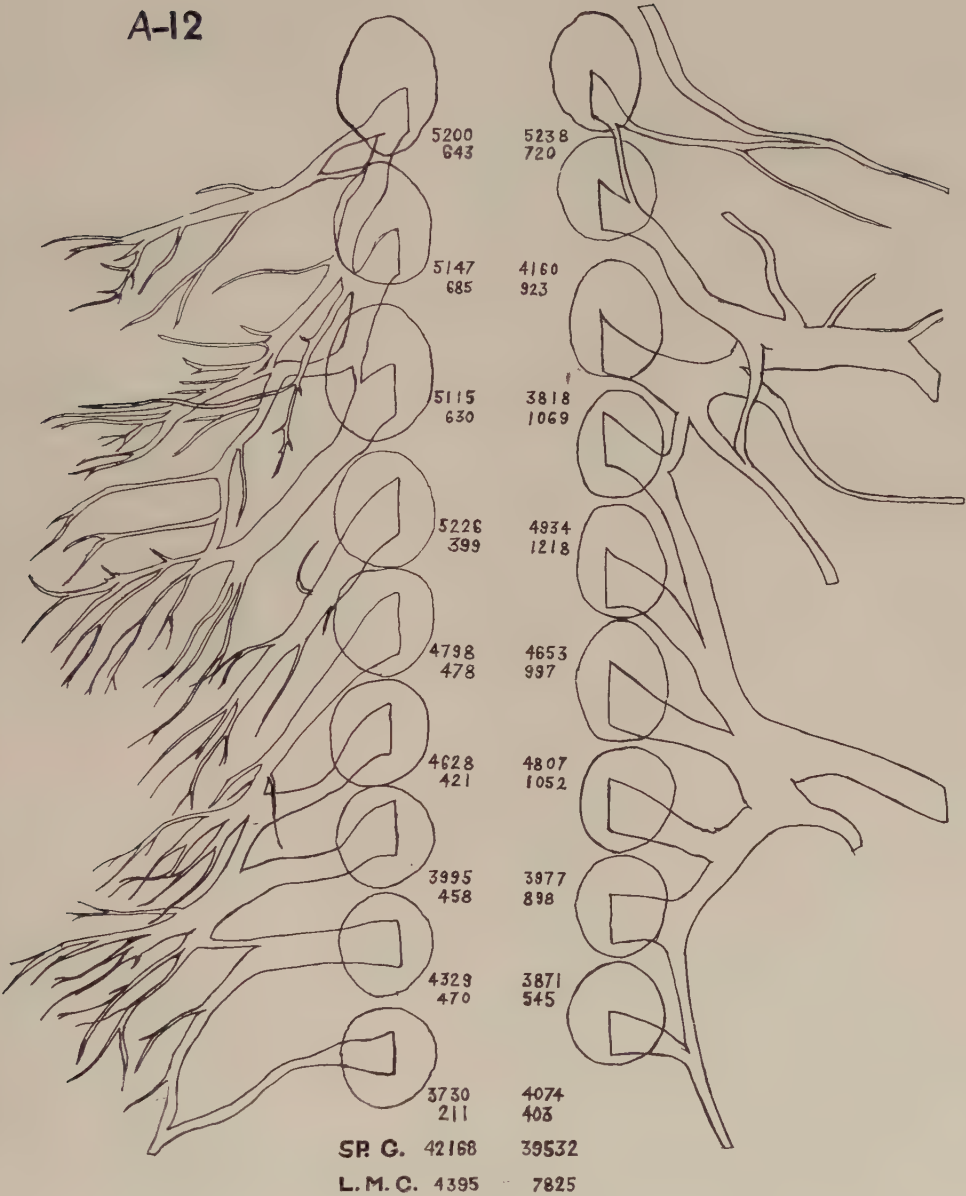
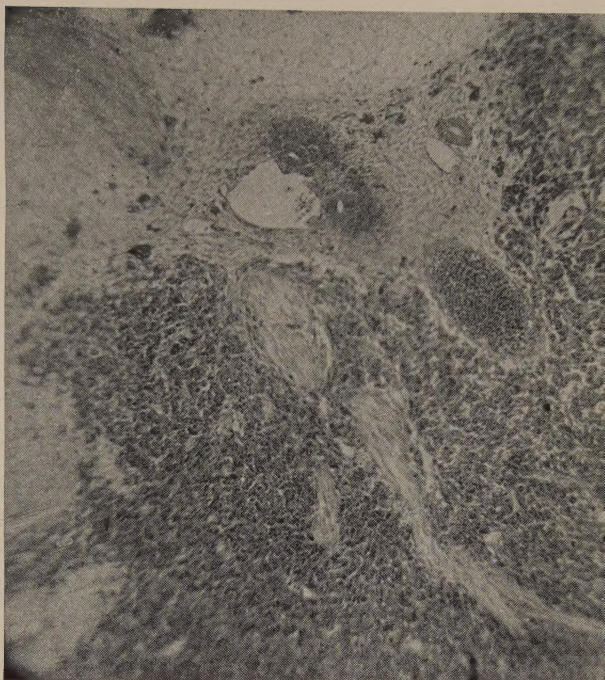
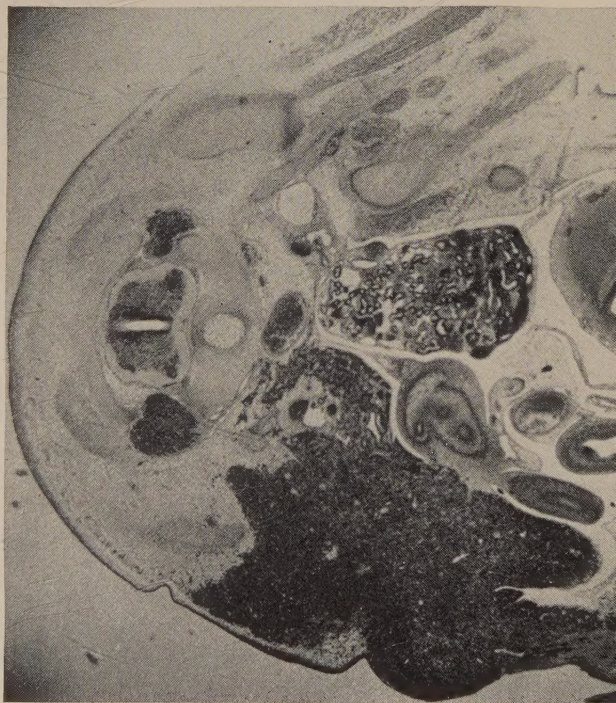
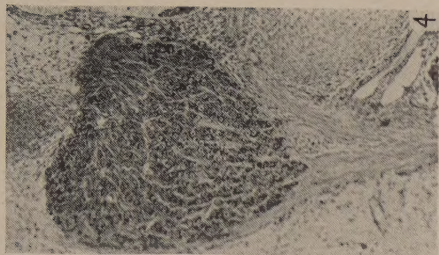
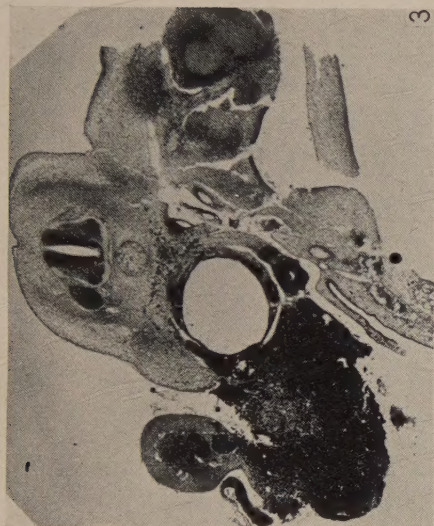
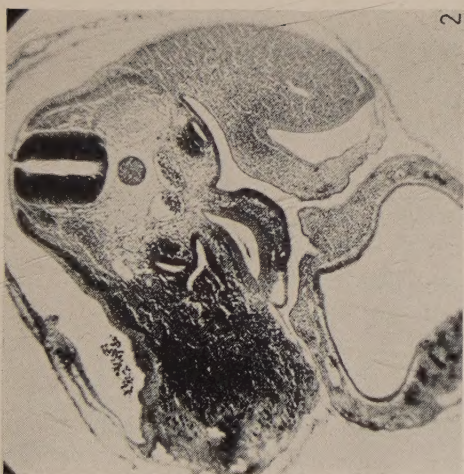


PLATE 2

EXPLANATION OF FIGURES

- 2 Cross section at lumbosacral level of A-1 (4 days' total incubation). Mouse sarcoma 180 transplant on left side.
- 3 Cross section at lumbosacral level of A-7 (6 days' total incubation). Mouse sarcoma 180 transplant on left side.
- 4 Section of an enlarged ganglion which innervated sarcoma 180 (case A-12).
- 5 Cross section of A-12 (8 days' total incubation) at spinal cord segment 27. Mouse sarcoma 180 on the left side.
- 6 Peripheral nerves of A-12 projecting into mouse sarcoma 180.



BOOK REVIEWS

DETAILED ATLAS OF THE HEAD AND NECK. By RAYMOND C. TRUEX and CARL E. KELLNER, xiii + 162 pages. 149 plates, 116 in color. $9\frac{1}{4} \times 12\frac{1}{2}$ inches. Oxford University Press, New York, 1948.

When one reads in the Foreword that the authors, an anatomist and an artist, labored in true symbiosis during the composition of this book, one cannot help remembering that this is an ancient tradition, going back 400 years to Vesalius and van Calcar. This latest product is not as pretentious nor as epoch making as the earlier one, but it is, nevertheless, a genuine product of the tradition because the drawings are all original and made from actual dissections. They are not copied or traced from photographs nor are they dressed-up diagrams from the author's imagination.

The book is divided into 4 parts. The first deals with regional anatomy and presents 82 drawings of dissections. The second gives the skeletal structures of the head and neck in figures 83-104; the third shows frontal sections, figures 105-116; and the fourth gives a series of cross sections in figures 117-136. In all appropriate parts, the association of the central nervous system with other structures of the head and neck is a prominent feature.

Doctor Truex has arranged the drawings of the dissections instructively and logically so that the reader may proceed from superficial to deeper structures in orderly sequence. As the title promises, this is a detailed atlas, but the selection of this detail has been made skillfully and does not interfere with its clarity. The book has already withstood a short period of practical test in the laboratory in which the students were able to find illustrations of practically all the structures that they encountered or sought in their dissections.

Mr. Kellner's skill as an artist shows up in the uniform excellence and clarity of the drawings. His ability to work with rapidity and precision must have contributed largely to the authors' unusual feat of completing an atlas in a relatively short time. Carl Kellner started his career as an artist in Baltimore at about the same time as Max Brödel and he enjoys recounting the story of how he won the first prize in a competition in which they both participated. During his long career he has illustrated many research publications and his drawings have been the outstanding contribution to the last three editions of Bailey's Histology. He has made a magnificent series of moulages of dissec-

tions and cross sections and he has contributed valuable technical advances in all phases of his graphic work. The reviewer is pleased to see that this versatile artist is at last receiving recognition.

The publishers are to be congratulated for the excellence of the reproduction of the plates, although their task must have been made somewhat easier by Mr. Kellner's skillful preparation of drawings for the individual color plates.

One lack which may be felt by both students and graduates in medicine is the depiction of the cervical fasciae in dissections. They are shown in the frontal and transverse sections, it is true, but these do not give the much needed pictures of continuity. It is not the place, in this review, to awaken the controversy on nomenclature, but one cannot resist questioning the desirability of some of the deviations from the B.N.A.

This is a beautiful and useful atlas and it is to be hoped that its authors may continue their effort and produce similar volumes on the rest of the body.

C. M. G.

LIVING ANATOMY: A PHOTOGRAPHIC ATLAS OF MUSCLES IN ACTION AND SURFACE CONTOURS. By R. D. LOCKHART. 71 pages. 149 figures. Faber and Faber, London.

This small book has as its stated purpose the kindling of enthusiasm for the living individual as a subject for anatomical study. It is intended to divert the student from his textual descriptions and laboratory specimens long enough to give him a preview of the application of his knowledge to the examination of a patient. In accordance with this purpose, the illustrations have been selected for their instructive qualities, interest and striking effect. Although the series is not intended to be complete, it includes illustrations of the position and action of nearly all the superficially placed muscles. Many of the poses are unusual and bring out individual muscles which are seldom seen dissociated from their action groups. Several different models have been used, sometimes with similar poses, to illustrate individual variation. Poses by models of both sexes afford instructive comparisons and enhance the value of the book for artists and students of physical education. The 149 photographs were selected from 700 negatives.

Any teacher or student of human anatomy must have as his final goal the visualization of anatomical relationships within the living individual. It is seldom practical, however, to employ a living model in classroom work and this book offers a convenient and instructive substitute.

C. M. G.